Overexpression, purification, crystallization and preliminary X-ray crystallographic analysis of SF173 from *Shigella flexneri*

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Shigella flexneri is a Gram-negative, anaerobic bacterium in the genus *Shigella* that can cause diarrhoea in humans. SF173, a hypothetical protein from *S. flexneri* 5a strain M90T, has been cloned, overexpressed, purified and crystallized as a part of laboratory-scale structural genomics project. The SF173 protein was crystallized using the sitting-drop vapour-diffusion method in the presence of 0.8 M succinic acid pH 7.0 at 293 K. Preliminary X-ray diffraction analysis revealed that the crystal diffracted to 1.47 Å resolution and belonged to space group *I*432, with unit-cell parameters $a = b = c = 110.245$ Å.

1. Introduction

Members of the genus *Shigella* are Gram-negative, obligate anaerobic bacteria that cause the most infectious of bacterial dysenteries, shigellosis (Wei *et al.*, 2003). This genus contains four species, including *Shigella flexneri*, which itself comprises 14 different serotypes and subserotypes. *Shigella* species permeate the colonic and rectal epithelium of many primates, including humans, causing the acute mucosal inflammation characteristic of shigellosis. In particular, *S. flexneri* is endemic in most developing countries and causes more fatalities than any other *Shigella* species (Bennish & Wojtyniak, 1991). Antibiotics can be used to treat shigellosis, as is shown by the reduction in bacterial secretion during antibiotic treatment. However, *S. flexneri* is increasingly becoming antibiotic-resistant (Ashkenazi *et al.*, 2003; Sansonetti, 2006). Owing to its importance as a human pathogen, the genomes of most strains of *Shigella*, including *S. flexneri*, have been completely sequenced (Onodera *et al.*, 2012). Despite the many protein structural studies available for other *Shigella* species, the literature is deficient in the structural assessment of proteins from *S. flexneri*. SF173 is one of the unknown proteins from *S. flexneri* strain M90T, and shows no sequence homology. Thus, precise analysis of the three-dimensional structure of SF173 is essential for its functional identification and will contribute to the structural genomics of *S. flexneri*. SF173 consists of 66 amino-acid residues with a molecular weight of 7.182 kDa, and the theoretical isoelectric point (pI) is 4.54. The secondary structure and protein crystallizability of SF173 were predicted using the *XtalPred* server. The prediction result shows that SF173 adopts a mainly α -helical structure (74%) and the combined crystallization class is 5. Here, we report the cloning, expression, purification, crystallization and preliminary X-ray analysis of SF173 from *S. flexneri* 5a strain M90T.

2. Materials and methods

2.1. Cloning, expression and purification

The ORF of SF173 was amplified by polymerase chain reaction (PCR) using the genomic DNA of *S. flexneri* 5a strain M90T as a template. The forward and reverse oligonucleotide primers were 5'-G GAA TTC **CAT ATG** GAA AAA TAT TGT GAG TTA ATA CGC-3' and 5'-CCG CCG **CTC GAG** TTC ATT GAC ATA AT-3', respectively, where the *Nde*I and *Xho*I restriction-enzyme cleavage sites are shown in bold. The reverse primer did not contain a stop codon. The PCR products were cleaved with *Nde*I and *Xho*I

and ligated into *NdeI/XhoI*-cleaved expression vector pET-21a (Novagen) in order to produce recombinant SF173 with a C-terminal hexahistidine (His_6) tag. The resulting construct contained eight non-native residues at the C-terminus (LEHHHHHH), including a His_6 tag that facilitates protein purification. The recombinant plasmid, which was verified by DNA sequencing, was transformed into *Escherichia coli* strain BL21(DE3) competent cells. The transformed cells were grown in Luria–Bertani (LB) medium containing 0.05 mg ml^{-1} ampicillin at 310 K. When the absorbance at 600 nm reached approximately 0.6, protein expression was induced by adding isopropyl β -D-1-thiogalactopyranoside (IPTG) to a final concentration of 0.5 mM. Protein expression was induced for 4 h. The cells were harvested by centrifugation and resuspended in lysis buffer (20 mM Tris–HCl, 500 mM NaCl, 10 mM imidazole, 5% glycerol, 10 mM β -mercaptoethanol, 0.2% NP-40 pH 7.2). The cells were disrupted by sonication and the supernatant was loaded onto a HisTrap FF column (GE Healthcare, USA) pre-equilibrated with lysis buffer. The bound protein was eluted using a gradient from 10 to 500 mM imidazole in the same buffer. Fractions that contained SF173 were pooled and dialyzed in buffer (20 mM Tris–HCl, 10 mM NaCl, 5% glycerol, 10 mM β -mercaptoethanol, 0.2% NP40 pH 7.2), and subsequently loaded onto a HiPrep DEAE FF anion-exchange column (GE

Healthcare). The bound protein was eluted using a linear NaCl gradient ($0.01\text{--}1 \text{ M}$) in the same buffer. Fractions containing SF173 were concentrated to about 2 ml and loaded onto a HiLoad 16/60 Superdex 200 column (GE Healthcare, USA) that had been equilibrated with the final buffer (20 mM Tris–HCl, 350 mM NaCl, 0.2 mM TCEP pH 7.2). The purified solution was concentrated to approximately 16.2 mg ml^{-1} , as estimated using the UV method (A_{280}) for protein determination, and used in crystallization screening trials. The purified protein was judged to be >95% pure by SDS–PAGE (Fig. 1).

2.2. Crystallization

SF173 was crystallized by the sitting-drop vapour-diffusion method in 96-well sitting-drop crystallization plates (Axygen) at 293 K. Initial crystallization conditions were screened by the sparse-matrix method (Jancarik *et al.*, 1991) using commercially available kits, including Index HT, PEG/Ion HT, Crystal Screen HT (Hampton Research, USA) and Wizard I, II, III and IV (Emerald Bio, USA). For screening, 0.5 μl protein solution was mixed with an equal volume of reservoir solution and equilibrated against 80 μl reservoir solution. Tiny crystals were obtained after 3 d in Wizard IV condition No. 13 consisting of 800 mM succinic acid pH 7.0. To obtain single crystals for X-ray diffraction, the initial crystallization conditions were further optimized by varying the concentration of protein and the volume of the protein/reservoir solution mixture using the sitting-drop vapour-diffusion method. The same volumes of protein solution (16.2 mg ml^{-1} in the same buffer) and reservoir solution (1 μl) were mixed and equilibrated against 80 μl reservoir solution in 96-well trays (Hampton Research, USA).

2.3. Data collection and processing

Prior to data collection, single crystals were equilibrated in the crystallization solution containing an additional 5% glycerol as a cryoprotectant and flash-cooled in liquid nitrogen. Diffraction data for the SF173 crystals were collected using an ADSC Q315r detector on the 5C SBII beamline at the Pohang Accelerator Laboratory (PAL), Pohang, Republic of Korea using 1° oscillations with a crystal-to-detector distance of 194.25 mm and a synchrotron X-ray beam of wavelength $1.0000 \text{ \AA}$. The crystal was exposed for 1 s per image. The data set for SF173 was collected to $1.5 \text{ \AA}$ resolution. The data sets were processed and scaled using *HKL-2000* (Otwinowski & Minor, 1997). The crystals belonged to space group *I432*, with unit-cell parameters $a = b = c = 110.245 \text{ \AA}$.

Figure 1
Purification of SF173. (a) Elution profile of purified SF173 from a gel-filtration column (43 ml void volume and 120 ml total bed volume). The flow rate was 1 ml min^{-1} and a single peak was observed for the purified protein solution. (b) SDS–PAGE of purified SF173 from fractions of the observed peak in gel-filtration chromatography. The highest peak fraction is in lane 4. Lane M contains molecular-weight marker (labelled in kDa).

Figure 2
Crystals of SF173. The average dimensions of the crystals were approximately $0.2 \times 0.15 \text{ mm}$.

Figure 3
X-ray diffraction pattern of an SF173 crystal.

Table 1
Summary of data-collection statistics.

Values in parentheses are for the highest resolution shell.

Diffraction source	5C SBII, PAL
Wavelength (Å)	1.00000
Temperature (K)	100
Detector	ADSC Q315r
Crystal-to-detector distance (mm)	194.25
Rotation range per image (°)	1
Total rotation range (°)	300
Exposure time per image (s)	1
Space group	I432
Unit-cell parameters (Å)	$a = b = c = 110.245$
Mosaicity (°)	0.487
Resolution range (Å)	50.00–1.47 (1.50–1.47)
Total No. of reflections	230509
No. of unique reflections	19783
Completeness (%)	96.8 (87.5)
Multiplicity	5.8 (5.2)
$\langle I/\sigma(I) \rangle$	49.7 (3.2)
$R_{p.i.m.}^\dagger$ (%)	2.6 (48.1)
$R_{merge}^\ddagger$ (%)	6.4 (65.1)
Overall B factor from Wilson plot (Å ²)	18.0

$^\dagger R_{p.i.m.}$ is defined as $\sum_{hkl} \{1/[N(hkl) - 1]\}^{1/2} \sum_i |I_i(hkl) - \langle I(hkl) \rangle| / \sum_{hkl} \sum_i I_i(hkl)$.

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

3. Results and discussion

Shigella species are important therapeutic targets as they cause infectious human diseases, and many researchers have focused on the function of essential proteins such as virulence factors in the pathogenesis of these bacteria (Hale, 1991; Jennison & Verma, 2004; Yang *et al.*, 2005; Deane *et al.*, 2006; Johnson *et al.*, 2006; Stevens *et al.*, 2006;

Xu *et al.*, 2013). However, studies of the relationship between protein structure and function are still lacking for *S. flexneri*. Specifically, only 3% of protein structures from *S. flexneri* have been deposited in the Protein Data Bank (118 out of 3820). In order to enrich the structural information on proteins expressed in *S. flexneri*, a laboratory-scale structural genomics project has been performed. As a part of the project, SF173 from *S. flexneri* 5a strain M90T was selected using bioinformatics tools. SF173 was cloned into a pET-21a(+) vector and successfully overexpressed in soluble form using an *E. coli* expression system. Protein of crystallographic-grade purity was obtained by Ni-NTA metal-affinity, anion-exchange and gel-filtration chromatography (Fig. 1). The yield of pure SF173 was approximately 15 mg per litre of culture. Crystals suitable for crystallographic analysis were grown by optimizing the initial condition and employing the sitting-drop vapour-diffusion method in 800 mM succinic acid pH 7.0. As shown in Fig. 2, cubic-shaped crystals were finally obtained of SF173 with dimensions of approximately $0.2 \times 0.2 \times 0.15$ mm. The crystals of SF173 diffracted to a resolution limit of 1.47 Å (Fig. 3). The statistics of the collected data are summarized in Table 1. In order to determine the crystal structure of SF173 using multiple-wavelength anomalous diffraction (MAD), selenomethione-labelled SF173 has been prepared and its crystallization is under optimization using similar crystallization conditions. The high-resolution crystal structure of SF173 will be valuable in elucidating the function of this protein.

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