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Purification, crystallization and preliminary X-ray diffraction analysis of the *Staphylococcus epidermidis* extracellular serine protease Esp

Esp, an extracellular serine protease from *Staphylococcus epidermidis*, has been shown to inhibit *S. aureus* biofilm formation and nasal colonization. The full-length 27 kDa pro-Esp was purified and digested with thermolysin to obtain mature Esp. The mature Esp containing 216 residues crystallized in space group $P2_1$, with unit-cell parameters $a = 39.5$, $b = 61.2$, $c = 42.5$ Å, $\beta = 98.2^\circ$ and one molecule in the asymmetric unit, with an estimated solvent content of 42%. A diffraction data set has been collected to 1.8 Å resolution on a rotating-anode home-source facility.

1. Introduction

Bacterial biofilms are increasingly being recognized as a major cause of persistent infections, particularly in patients receiving medical implants such as ventilation tubes, in-dwelling catheters, artificial heart valves *etc.* After initial adhesion and invasion, biofilm formation is the key step involved in *Staphylococcus epidermidis* virulence during device-associated infections (Rohde *et al.*, 2007, 2010; Mack *et al.*, 2006). Biofilms are composed of extracellular polysaccharides, proteins and other nutrients essential for microbial survival. Pathogens embedded in such biofilms are less susceptible than their planktonic counterparts to antibiotics and biocides (Gilbert *et al.*, 2002; Foster, 2005), and are also impervious to destructive inflammatory processes of the host (Vuong *et al.*, 2004). Generally, Gram-positive bacteria are less susceptible to human complement, probably because of their thick peptidoglycan layer. However, human polymorphonuclear neutrophils (PMNs) can eliminate microbes when brought into close contact (Vuong *et al.*, 2004). Interestingly, a typical biofilm of *S. epidermidis* can activate the complement better than its planktonic colleagues, as shown by measurement of the released anaphylotoxin C3a (Kristian *et al.*, 2008). However, deposition of C3b on the target cells was diminished in biofilm-embedded *S. epidermidis*, which would contribute to its escape from PMN killing, in addition to complement evasion (Kristian *et al.*, 2008).

Bacteria, which were once thought of as organisms that rarely interact, have recently been found to lead highly social lives in biofilms (West *et al.*, 2006). Central to such cooperative living is their ability to detect the local cell density and coordinate their behaviors (Bassler & Losick, 2006). This detection ability, termed quorum sensing, involves the secretion and detection of auto-inducer molecules that accumulate in the biofilm in proportion to the respective cell density. Based on the auto-inducer density, microbes modulate behaviors such as surface attachment (Dunne, 2002), the production of 'sticky' extracellular polymeric substances that are conducive to the transfer of genetic material (Sakuragi & Kolter, 2007), sporulation (Grossman, 1995) and secretion of virulence factors (Miller *et al.*, 2002; Zhu *et al.*, 2002). In contrast to such cooperative abilities of microbes, Iwase *et al.* (2010) have recently identified a staphylococcal species that exhibits an ability to regulate the survival of other classes of pathogens in biofilms (Iwase *et al.*, 2010).

S. epidermidis, a Gram-positive commensal bacterium, is part of the normal human flora and is a dominant colonizer of the upper respiratory tract, especially the nostrils (Iwase *et al.*, 2010). The nostrils are also primary reservoirs for another human bacterial pathogen, *S. aureus*, which is responsible for illnesses ranging from

minor skin infections (e.g. pimples, impetigo and boils) to life-threatening diseases such as pneumonia, endocarditis and meningitis. The recent emergence of methicillin-resistant and vancomycin-resistant *S. aureus* has been identified as being responsible for a large number of infections and deaths all over the world (Klein *et al.*, 2007; Lowy, 1998; Hiramatsu *et al.*, 1997), necessitating an urgent need for identifying new therapies that do not elicit resistance. Understanding the mode of *S. aureus* biofilm inhibition by *S. epidermidis* could open an avenue for an alternative therapeutic strategy.

Epidemiological studies on staphylococcal nasal colonization in the nasal cavities of volunteers have revealed the presence of both *S. epidermidis* and *S. aureus* in some populations, and the presence of *S. epidermidis* along with the absence of *S. aureus* in many others (Iwase *et al.*, 2010). Further co-culturing in the nasal cavities helped Iwase *et al.* (2010) to identify two types of *S. epidermidis*: one type can cohabit with *S. aureus*, while the other inhibits the growth of the latter in a dose-dependent manner (Iwase *et al.*, 2010). More interestingly, culture supernatants of inhibitory *S. epidermidis* destroyed previously formed *S. aureus* biofilm, while the non-inhibitory-type cell culture had no effect.

Iwase *et al.* (2010) isolated a 27 kDa protein from the inhibitory-type *S. epidermidis* cell culture and identified it as a serine protease, previously suggested to be pro-Esp from oral strains of *S. epidermidis* (Moon *et al.*, 2001; Dubin *et al.*, 2001). The pro-Esp, also previously known as SspA or GluSE (Ohara-Nemoto *et al.*, 2002; Moon *et al.*, 2001), contains a 66-residue pro-peptide at the N-terminal end and a mature 216-residue polypeptide in the C-terminal region (Fig. 1). Mature Esp processed from pro-Esp by an unknown protease exhibits significant sequence similarity (59.1%) to *S. aureus* V8 protease (Ohara-Nemoto *et al.*, 2002). There is limited similarity in the pro-peptide segments (<25%) of the Esp and V8 serine proteases, and the mature Esp lacks 55 residues that comprise the multiple PNN/PDN tripeptides observed in the C-terminus of active V8 (Ohara-Nemoto *et al.*, 2002). In this paper, we report the cloning, purification, activation, crystallization and preliminary crystallographic characterization of mature Esp. The mature Esp crystals diffracted to 1.8 Å resolution using a rotating-anode home-source facility and the diffraction data collected were of good quality and were suitable for structure determination.

2. Materials and methods

2.1. Expression and purification

S. epidermidis JK16 strain was obtained from the American Type Culture Collection (ATCC). The primers Esp-pET-*Nde*II (5'-CATATGAAAAGAGATTTTATCTATATG-3') and Esp-pET-*Bam*HI (5'-GGATCCTTACTGAATATTTATATCAG-3') harboring *Nde*I and *Bam*HI restriction sites, respectively, were used to PCR-amplify the sequence of *esp* (coding Met1–Gln282) from chromosomal DNA of *S. epidermidis* strain ATCC 12228 (gene accession No. AAO05142.1), which was cloned into the pET28b plasmid (EMD

Biosciences/Novagen). The resulting pET28-His-proEsp plasmid, which includes a His₆ tag at the N-terminus, was transformed into *Escherichia coli* BL21 (DE3) cells.

Overnight cultures (10 ml) of stationary-phase *E. coli* were used to inoculate 1.01 Luria–Bertani (LB) broth supplemented with 100 µg ml^{−1} ampicillin. The cells were allowed to grow at 310 K until the culture reached an OD_{600 nm} of ~0.6. Protein expression was induced by the addition of isopropyl β-D-1-thiogalactopyranoside (IPTG) to a final concentration of 1.0 mM and the culture was incubated overnight at 303 K. The cells were harvested by centrifugation at 2568g (Beckman Allegra 6R Centrifuge) for 20 min at 277 K and were resuspended in lysis buffer [50 mM sodium phosphate pH 7.5, 300 mM NaCl, EDTA-free protease-inhibitor cocktail tablet (Roche), 10%(v/v) glycerol, 5 mM imidazole]. The cells were lysed using a French press (76 MPa) and the soluble supernatant was collected by centrifugation at 18 384g for 20 min at 277 K. The supernatant was loaded onto a column containing 8 ml Ni-NTA Superflow Agarose resin pre-equilibrated with lysis buffer. The column was washed with ten bed volumes of buffer A (50 mM sodium phosphate pH 7.4, 300 mM NaCl) and the bound protein was eluted with a linear gradient of 0–100% buffer B (50 mM sodium phosphate pH 7.4, 300 mM NaCl, 300 mM imidazole). Fractions containing the pro-Esp, as determined by SDS–PAGE, were pooled.

In light of the high sequence similarity and similar enzymatic properties of *S. epidermidis* Esp and *S. aureus* V8, the metallo-protease thermolysin employed for the activation of pro-V8 was used for cleavage of the (Ser66–Val67) pro-Esp N-terminal pro-peptide (Yoshikawa *et al.*, 1992; Ohara-Nemoto *et al.*, 2002). The N-terminal His₆-tag-fused pro-Esp was digested by thermolysin at 310 K for 4 h and the proteolysis was stopped by the addition of 5 mM EDTA; the cleavage was confirmed by SDS–PAGE. The proteolytic activity of mature Esp was tested using an asocasein assay (Sugimoto *et al.*, 2011) as follows: the product of thermolysin-treated recombinant protein was diluted into a reaction mixture consisting of 1% asocasein, 100 mM Tris–HCl pH 8.0, 5 mM EDTA. After incubation at 310 K for 5 h, an equal volume of 5% trichloroacetic acid (TCA) was added to stop the reaction. The soluble material was recovered by centrifugation at 2123g for 10 min and the absorbance was measured at 400 nm.

The mature Esp was further purified by gel-filtration chromatography on a Superdex 75 10/30 column (GE Healthcare) in buffer consisting of 20 mM Tris–HCl pH 7.2, 150 mM NaCl. For crystallization, the protein was concentrated to 22 mg ml^{−1} using an Amicon ultrafiltration system.

2.2. Crystallization

Initial crystallization conditions were screened at 293 K by the sitting-drop vapor-diffusion method with the commercial crystallization screens Crystal Screen HT, Index HT (Hampton Research) and Wizard Screens I and II (Emerald BioSystems) using a Crystal Phoenix robot (Art Robbins Instruments) and 96-well Intelli-Plates (Art Robbins). Each crystallization drop was prepared by mixing 0.2 µl reservoir solution and 0.2 µl protein solution. Crystals were observed in several conditions after a week and the conditions were optimized manually with the help of laboratory-made solutions and using the hanging-drop vapor-diffusion method. Crystals used for diffraction data collection were obtained from drops consisting of equal volumes (1 µl) of protein solution (14 mg ml^{−1} in 20 mM Tris–HCl buffer pH 7.2, 150 mM NaCl) and well solution (1 µl) that were equilibrated against a 1 ml reservoir. Thin rod-shaped crystals (Fig. 2)

Figure 1
A schematic diagram of pro-Esp. Pro-Esp is composed of signal peptide (SP; Met1–Ala27), pro-peptide (PP; Lys28–Ser66) and the following mature Esp (Val67–Gln282). The arrow at the top indicates the thermolysin cleavage site.

obtained from a condition with 0.25 M potassium acetate, 22%(w/v) PEG 3350 in the well were used for data collection.

2.3. Data collection and processing

Native diffraction data were collected to 1.8 Å resolution (Fig. 3) using an R-Axis IV imaging-plate detector mounted on an in-house Rigaku rotating-anode X-ray generator operating at 100 mA and 50 kV. Diffraction data were collected from a single crystal over a range of 360° in 1° steps at a 130 mm crystal-to-detector distance and

Figure 2

Crystals of mature Esp obtained by the hanging-drop vapor-diffusion method using 0.25 M potassium acetate and 22% PEG 3350 as precipitants at room temperature. The dimensions of the largest crystal in the drop are $0.5 \times 0.04 \times 0.02$ mm.

Figure 3

X-ray diffraction image of a native mature Esp crystal collected using an R-Axis IV imaging-plate detector mounted on an in-house Rigaku rotating-anode X-ray generator operating at 100 mA and 50 kV.

Table 1

Data-collection and processing statistics for mature Esp.

Values in parentheses are for the highest resolution shell.

No. of crystals	1
Beamline	In-house
Wavelength (Å)	1.5418
Detector	R-Axis IV image plate
Crystal-to-detector distance (mm)	130
Rotation range per image (°)	1
Total rotation range (°)	360
Exposure time per image (s)	300
Resolution range (Å)	40–1.8 (1.86–1.80)
Space group	$P2_1$
Unit-cell parameters (Å, °)	$a = 39.5$, $b = 61.2$, $c = 42.5$, $\beta = 98.2$
Mosaicity (°)	0.3
Total No. of measured intensities	132840
Unique reflections	17525
Multiplicity	7.6 (6.8)
Mean $I/\sigma(I)$	16.1 (4.3)
Completeness (%)	94.2 (86.9)
χ^2	0.94 (1.32)
$R_{\text{merge}}^{\dagger}$ (%)	5.6 (29.1)
Overall Wilson B factor (Å ²)	24.3
No. of molecules in the asymmetric unit	1

$\dagger R_{\text{merge}} = \sum_{hkl} \sum_i |I_i(hkl) - \langle I(hkl) \rangle| / \sum_{hkl} \sum_i I_i(hkl)$, where $I_i(hkl)$ are the intensities of symmetry-related reflections and $\langle I(hkl) \rangle$ is the average intensity over all observations.

with 5 min exposure per frame. The cryoprotectant was prepared by mixing 20 µl 100%(v/v) ethylene glycol and 80 µl well solution consisting of 0.25 M potassium acetate, 22%(w/v) PEG 3350. The selected crystal was picked up using a small nylon loop and swiped through the prepared cryoprotectant solution before being positioned in the liquid-nitrogen stream and X-ray beam. The diffraction data were processed with *d*TREK* (Pflugrath, 1999) and the crystallographic data statistics are presented in Table 1.

The native mature Esp crystals belonged to the monoclinic space group $P2_1$, with unit-cell parameters $a = 39.5$, $b = 61.2$, $c = 42.5$ Å, $\beta = 98.2^\circ$. Matthews coefficient calculations (Matthews, 1968) suggested the presence of one molecule in the asymmetric unit (calculated V_M of $2.14 \text{ Å}^3 \text{ Da}^{-1}$, estimated solvent content of 42%).

3. Results and discussion

The pro-Esp of *S. epidermidis* was purified and activated by thermolysin to obtain mature Esp. Single crystals of mature Esp suitable for diffraction studies were obtained in a week from a condition containing a mixture of 0.25 M potassium acetate and 22%(w/v) PEG 3350 as a precipitant. The Esp crystals diffracted to 1.8 Å resolution and belonged to the monoclinic space group $P2_1$. The V_M calculation suggested that the asymmetric unit is likely to contain one molecule, with a solvent content of 42%. Attempts to solve the crystal structure of mature Esp by molecular replacement using the coordinates of *S. aureus* V8 (PDB entry 2o8l; Prasad *et al.*, 2004) as the starting model have not been successful. Attempts to use a selenomethionine derivative for MAD/SAD phasing are in progress; there are two suitable methionine residues for replacement with selenomethionine in mature Esp.

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