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A preliminary X-ray study of sedoheptulose-7-phosphate isomerase from *Burkholderia pseudomallei*

Mi-Sun Kim and Dong Hae Shin*

College of Pharmacy, Division of Life and Pharmaceutical Sciences, Ewha Womans University, Seoul 120-750, Republic of Korea

Correspondence e-mail: dhshin55@ewha.ac.kr

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Sedoheptulose-7-phosphate isomerase (GmhA) converts D-sedoheptulose 7-phosphate to D,D-heptose 7-phosphate. This is the first step in the biosynthesis pathway of NDP-heptose, which is responsible for the pleiotropic phenotype. This biosynthesis pathway is the target of inhibitors to increase the membrane permeability of Gram-negative pathogens or of adjuvants working synergistically with known antibiotics. *Burkholderia pseudomallei* is the causative agent of melioidosis, a seriously invasive disease in animals and humans in tropical and subtropical areas. GmhA from *B. pseudomallei* is one of the targets of antibiotic adjuvants for melioidosis. In this study, GmhA has been cloned, expressed, purified and crystallized. Synchrotron X-ray data were also collected to 1.9 Å resolution. The crystal belonged to the primitive orthorhombic space group $P2_12_12_1$, with unit-cell parameters $a = 61.3$, $b = 84.2$, $c = 142.3$ Å. A full structural determination is under way in order to provide insights into the structure–function relationships of this protein.

1. Introduction

The major component of the outer membrane of Gram-negative bacteria is lipopolysaccharide (LPS), which is composed of lipid A, a conserved core oligosaccharide region and an O-specific polysaccharide chain or O antigen (Raetz, 1996). LPS has an important role in maintaining the structural integrity of the bacterial outer membrane. The core oligosaccharide can be subdivided into an outer core generally composed of hexoses and hexosamines and an inner core comprised of 3-deoxy-D-*manno*-oct-2-ulosonic acid and L,D-heptose units that are essential for viability. *Escherichia coli* K-12 mutants lacking heptoses in the LPS display pleiotropic phenotypes owing to reduced stability of the outer membrane. These phenotypes are characterized by an extreme sensitivity to novobiocin, detergents and bile salts (Tamaki *et al.*, 1971). The first step of the biosynthesis pathway of NDP-heptose in the inner core responsible for the pleiotropic phenotype is sedoheptulose-7-phosphate isomerization, which is catalyzed by sedoheptulose-7-phosphate isomerase (GmhA; Eidels & Osborn, 1974). This biosynthesis pathway is the target for the future development of inhibitors that would increase the membrane permeability of Gram-negative pathogens or of adjuvants working synergistically with known antibiotics. The crystal structures of GmhA from *E. coli* and *Pseudomonas aeruginosa* have been reported in apo, substrate-bound and product-bound forms (Taylor *et al.*, 2008). Structural comparison showed that during the substrate-isomerization process GmhA adopts two distinct 'open' and 'closed' conformations through reorganization of its quaternary structure.

Burkholderia pseudomallei is notorious for its pathogenicity, causing melioidosis (Wiersinga *et al.*, 2006). Since mortality from melioidosis septic shock remains high despite appropriate antimicrobial therapy, the development of more effective antibiotics or adjuvants is awaited. It is also classified as a potential bioterrorism agent owing to its rareness in Western countries, high accessibility, environmental persistence, the high prevalence of severe sepsis and septic shock mortality and the fact that there is no vaccine available (Gilad *et al.*, 2007). A genomic sequence search using the *BLAST* web server (http://www.ncbi.nlm.nih.gov) shows that *B. pseudomallei*

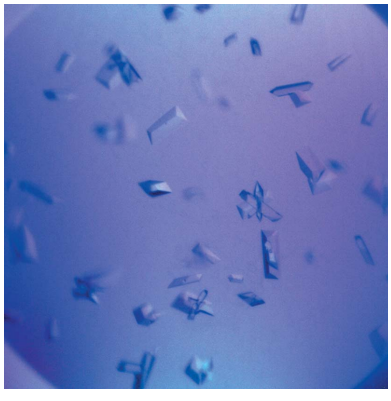

Table 1
Data-collection statistics.

Values in parentheses are for the highest resolution shell.

X-ray source	Pohang Light Source 4A
X-ray wavelength (Å)	1.000
Temperature (K)	100
Space group	$P2_12_12_1$
Unit-cell parameters	
a (Å)	61.3
b (Å)	84.2
c (Å)	142.3
Resolution range (Å)	50.0–1.90 (1.97–1.90)
Unique reflections	56579 (4369)
$R_{\rm merge}$† (%)	9.6 (49.9)
Data completeness (%)	96.1 (75.1)
$\langle I/\sigma(I)\rangle$	18.6 (2.3)

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

has the NDP-heptose biosynthesis pathway. An open reading frame of *B. pseudomallei* codes for a GmhA, the enzyme catalyzing the first step in this pathway, of 20.8 kDa.

We have initiated the determination of the three-dimensional structure of GmhA from *B. pseudomallei* to provide a structural template for the development of antibiotic adjuvants as anti-melioidosis agents. Here, we report the cloning, overexpression, purification, crystallization and preliminary X-ray study of this enzyme.

2. Experimental methods

2.1. Cloning of GmhA

The primers (Genotech, DaeJeon, Republic of Korea) prepared for ligation-independent cloning (LIC) of GmhA were 5′-GGCGGTGGTGGCGGCATGGAGAATCGCGAATTGACGTACAT-3′ for the forward primer and 5′-GTTCTTCTCCTTTGCGCCCCTACTGCTTCCCGAAAATGGAGTGCT-3′ for the reverse primer. The GmhA gene was amplified by PCR using 200 ng *B. pseudomallei* genomic DNA template and 0.5 µ*M* of primers. PrimeSTAR HS DNA polymerase with GC buffer (Takara Bio Inc., Shiga, Japan) designed for high-GC-content genomic DNA was used. The amplified LIC expression vector pB2 (Kim *et al.*, 2005), a derivative of pET21a vector (Novagen, Madison, Wisconsin, USA), was incubated with T4 DNA polymerase (New England Biolabs, Beverley, Massachusetts, USA) in the presence of 1 m*M* dATP at 310 K for 30 min, followed by a 343 K incubation for 20 min. The amplified PCR product was prepared for vector insertion using the same protocol as described above, except for the presence of 1 m*M* dTTP instead of 1 m*M* dATP. The prepared insert was annealed into the pB2 vector that expresses the cloned gene by fusing it to a noncleavable N-terminal His_6 tag and transforming it into DH5α competent cells to obtain fusion clones. Clones were screened by sequencing of plasmid DNA and transformed into *E. coli* strain BL21 (DE3) for protein expression (Kim *et al.*, 1998).

2.2. Protein expression and purification

E. coli BL21 (DE3) transformed with the cloned vector that harbours the GmhA gene was grown on Luria–Bertani (LB) agar plates containing 150 µg ml^{-1} ampicillin. Several colonies were picked and grown in capped test tubes with 10 ml LB broth containing 150 µg ml^{-1} ampicillin. A cell stock was prepared with 0.85 ml culture and 0.15 ml glycerol and frozen at 193 K for preparation of a large culture. The frozen cell stock was grown in 5 ml LB medium and diluted into 500 ml fresh LB medium. The culture was incubated at 310 K with shaking until an OD_{600} of 0.6–0.8 was reached. At this point, GmhA was induced with isopropyl β-D-1-thiogalactopyranoside (IPTG) at a final concentration of 1 m*M*. The culture was grown at 310 K for a further 3 h in a shaking incubator. The cells were harvested by centrifugation at 6500 rev min^{-1} for 10 min in a high-speed refrigerated centrifuge (Supra 22K, Hanil Science Industrial, Republic of Korea). The cultured cell paste (1.5 g) was resuspended in 12.5 ml buffer consisting of 50 m*M* Tris–HCl pH 8.0, 100 m*M* NaCl, 10 m*M* imidazole, 1 m*M* PMSF, 10 µg ml^{-1} DNase I and Roche protease-inhibitor cocktail (Roche Applied Science, Indianapolis, Indiana, USA). The cell suspension was disrupted using a Digital Sonifier 450 (Branson Ultrasonics Co., Danbury, Connecticut, USA). The cell debris was pelleted by centrifugation at 15 000 rev min^{-1} for 30 min in a high-speed refrigerated centrifuge (Supra 22K, Hanil Science Industrial, Republic of Korea). The supernatant was purified using a HisTrap affinity column mounted on an ÄKTA Explorer (GE Healthcare, Piscataway, New Jersey, USA). The column was equilibrated with a buffer consisting of 50 m*M* Tris–HCl pH 8.0, 300 m*M* NaCl and 10 m*M* imidazole. The target protein was eluted with a buffer consisting of 50 m*M* Tris–HCl pH 8.0 and 100 m*M* NaCl with a gradient from 10 to 500 m*M* imidazole. GmhA was further purified by ion-exchange chromatography using a 5 ml Hi-Trap Q column (GE Healthcare, Piscataway, New Jersey, USA) equilibrated with buffer containing 50 m*M* Tris–HCl pH 8.0. The protein was eluted at 0.4 *M* NaCl using a linear gradient to 1.0 *M* NaCl. SDS–PAGE showed one band around 22 kDa, corresponding to the molecular weight of GmhA. The purified protein contained a noncleavable N-terminal His_6 tag followed by six glycine residues. The protein was concentrated to 20 mg ml^{-1} for crystallization in a buffer consisting of 0.4 *M* NaCl and 50 m*M* Tris–HCl pH 8.0. Dynamic light scattering (DynaPro 99, Proterion Corporation, Piscataway, New Jersey, USA) showed a single monodisperse peak corresponding to the approximate size of tetrameric GmhA.

2.3. Crystallization

Screening for crystallization conditions was performed using the sparse-matrix method (Jancarik & Kim, 1991) with several screens from Hampton Research (Hampton Research, Laguna Niguel, California, USA). A Hydra Plus One crystallization robot (Matrix Technologies, Hudson, New Hampshire, USA) was used to set up the screens using the sitting-drop vapour-diffusion method at room temperature. A 96-well Intelli-Plate (Art Robbins Instrument, Salt Lake City, Utah, USA) was used; sitting drops were made by mixing 0.2 µl protein solution and 0.2 µl reservoir solution and were equilibrated over 90 µl reservoir solution. The VDX48 plate (Hampton Research, Laguna Niguel, California, USA) was used to optimize the crystallization conditions; hanging drops were made by mixing 0.8 µl protein solution and 0.8 µl reservoir solution containing 2.8 *M* sodium acetate pH 7.0 and were equilibrated over 100 µl reservoir solution.

2.4. Data collection and reduction

A concentrated magnesium acetate solution was added to the well solution to 1.75 *M* magnesium acetate concentration and the sitting drop was allowed to equilibrate for 1 min. The drop was flash-frozen in liquid nitrogen and exposed to X-rays. X-ray diffraction data sets were collected at a single wavelength on beamline 4A at the Pohang Light Source using a Quantum 4 CCD detector (Area Detector System Co., Poway, California, USA) placed 300 mm from the sample. The oscillation range per image was 1.0°, with no overlap between two contiguous images. X-ray diffraction data were

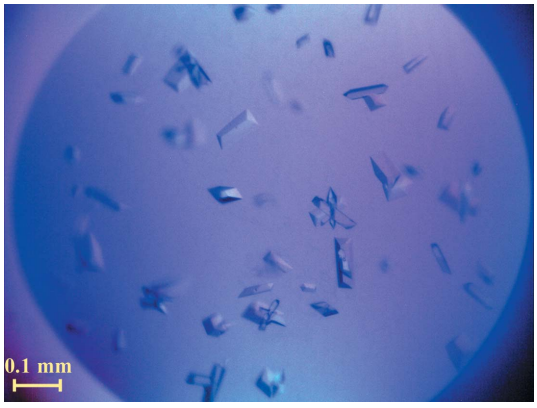

Figure 1
Rod-shaped crystals. These crystals appeared in the condition 2.8 *M* sodium acetate pH 7.0. The crystals grew to approximate dimensions of 0.1 × 0.05 × 0.05 mm in a week.

processed and scaled using the *HKL*-2000 and *SCALEPACK* programs (Otwinowski & Minor, 1997). The synchrotron data were collected to 1.9 Å resolution. Data statistics are summarized in Table 1.

3. Results and discussion

The yield of purified protein was ∼20 mg per litre of *E. coli* culture. After performing anion-exchange chromatography, GmhA appeared to be approximately 99% pure, with a prominent protein band at around 22 kDa on SDS–PAGE. The first crystallization trial produced rod-shaped crystals that appeared under various conditions using PEG 3350, PEG 4000, 2-propanol or salts as precipitants. However, diffraction-quality crystals were obtained using a reservoir solution consisting of 2.8 *M* sodium acetate pH 7.0. Crystals grew to dimensions of 0.1 × 0.05 × 0.05 mm within a week at 296 K (Fig. 1).

Synchrotron data were collected to 1.9 Å resolution. The X-ray diffraction data were processed and scaled using *HKL*-2000 (Otwinowski & Minor, 1997). The crystal belonged to the primitive orthorhombic space group $P2_12_12_1$, with unit-cell parameters $a = 61.3$, $b = 84.2$, $c = 142.3$ Å. Assuming that the asymmetric unit contains four GmhA molecules, a corresponding Matthews coefficient V_M of 2.08 Å^3 Da^{-1} and a solvent content of 38.6% (Matthews, 1968) were obtained. Details of the data-collection statistics are presented in Table 1. The structure will be solved by molecular replacement using GmhA from *E. coli*, which has a sequence homology of 72% to our target molecule, as a search model.

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