Biodesign

pISSN 2288-6982 | eISSN 2288-7105

CRYSTALLIZATION P 40-43

Crystallization and preliminary crystallographic analysis of IlvC, a ketol-acid reductoisomerase, from *Streptococcus pneumoniae*

Gyuhee Kim and Sangho Lee*

Department of Biological Sciences, Sungkyunkwan University, 2066 Seobu-ro, Suwon, Gyeonggi 16419, Korea.

*Correspondence: sangholee@skku.edu

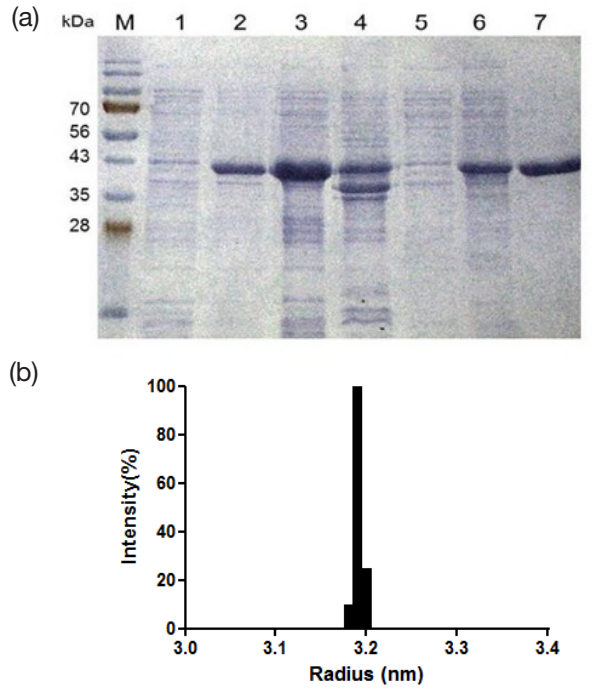
IlvC, a ketol-acid reductoisomerase, plays a critical role in alkyl migration and catalyzes the second step in the biosynthesis of branched amino acids such as leucine, valine and isoleucine. As an initial step to investigate whether IlvC is involved in pneumococcal growth and virulence from the structural background, *ilvC* from *Streptococcus pneumoniae* D39 (SpIlvC) was cloned and overexpressed in *Escherichia coli*. Crystals of SpIlvC were obtained by hanging-drop vapour diffusion in 0.1 M HEPES pH 7.5, 0.1 M NaCl, 1.5 M ammonium sulfate and diffracted to 1.69 Å resolution. The SpIlvC crystal belonged to space group $P2_12_12_1$ with unit cell parameters a = 69.1°, b = 104.3°, c = 110.9° and contained two molecules in the asymmetric unit.

INTRODUCTION

The biosynthetic pathway of branched chain amino acids (BCAAs) such as isoleucine, leucine, and valine, is present in bacteria, algae, plants, fungi and archaea but not in mammals, thereby rendering enzymes of this pathway good targets for intervention (McCourt et al., 2006). Interestingly, the BCAA biosynthetic pathway is reportedly involved in respiratory tract pathogens such as *Actinobacillus pleuropneumoniae* (Subrashchandrabose et al., 2009). *A. pleuropneumoniae ilvI* encodes acetohydroxyacid synthase and its mutant showed defects in BCAA synthesis, inhibited growth and reduced virulence in swine. IlvE, branched chain amino acids aminotransferase, from *Sterptococcus mutants* is involved in acid tolerance as well as its catalytic activity in the BCAA pathway (Santiago et al., 2012). Ketol-acid reductoisomerase (KARI; EC 1.1.1.86) catalyzes the conversion of acetohydroxy acids such as 2-acetolactate or 2-aceto-2-hydroxybutyrate to dihydroxy valerates including 2,3-dihydroxy-3-isovalerate or 2,3-dihydroxy-3-methylvalerate, the second step in the BCAA biosynthetic pathway (McCourt et al., 2006). KARIs promote alkyl migration and NADPH-dependent reduction in the presence of NADPH and magnesium ions (Dumas et al., 2001). Although KARI structures from some bacteria and plants have been determined, only one structure from *Escherichia coli* is available in both apo and holo forms (Tyagi et al., 2005; Wong et al., 2012). *Streptococcus pneumoniae* is a pathogenic Gram-positive bacterium causing a global health problem (Kadioglu et al., 2008). IlvC from *Streptococcus pneumoniae* (SpIlvC) is a pneumococcal KARI. Despite the involvement of the BCAA pathway in pneumococcal diseases, the reaction mechanism of IlvC in *S. pneumoniae* (SpIlvC) and its role in pneumococcal dieases is not well understood. To investigate whether SpIlvC affects the growth and virulence of *Streptococcus pneumoniae* via the BCAA pathway, we attempted to crystallize SpIlvC and to analyze diffraction pattern of the SpIlvC crystal.

RESULTS AND DISCUSSION

To investigate roles of SpIlvC in the growth and virulence of *S. pneumoniae*, SpIlvC was expressed in *E. coil* and purified to homogeneity by affinity chromatography followed by size-exclusion chromatography. Purification steps of SpIlvC were monitored by SDS-PAGE (Figure 1a). Concentrated to 11.5 mg ml^{-1} of SpIlvC was monodisperse in solution judged by dynamic light scattering (Figure 1b). Initial crystallization screening by microbatch method yielded clusters of plate-shaped crystals that diffracted poorly and suffered nonmerohedral twinning in diffraction patterns. Subsequent refinement of the initial crystallization condition by hanging drop method led to diffraction-quality crystals in dagger shapes (Figure 2). The SpIlvC crystals appeared in three weeks only after formation of precipitates. The SpIlvC crystal diffracted to 1.69 Å resolution (Figure 3). The SpIlvC crystal belonged to the space group $P2_12_12_1$ with unit cell parameters a = 69.1 Å, b = 104.3 Å and c = 110.9 Å. Data collection and processing statistics are summarized in Table 3. The Matthews coefficient (V_M) was calculated to be 2.66 $Å^3$ Da^{-1}, with a solvent content of 53.83 % (Matthews et al., 1968) assuming a dimer in the asymmetric unit. For phasing by molecular replacement (MR), a MR solution was obtained with Phaser in the *PHENIX* suite (Adams et al., 2010)



	R_H (nm)	Diameter (nm)	% Pd	% Intensity
SpIlvC	3.19	6.39	11.5	100

FIGURE 1 | (a) SDS-PAGE for expression and purification of SpIlvC. Lane M: protein molecular mass markers (labelled in kDa); lane 1, uninduced cell lysate; lane 2, induced cell lysate; lane 3, supernatant; lane 4, cell pellet; lane 5, flow- through from Ni-NTA resin; lane 6, elution of SpIlvC from Ni-NTA resin; and lane 7, SpIlvC after size exclusion chromatography. (b) Dynamic light scattering of the purified SpIlvC shows that SpIlvC is monodisperse in solution.

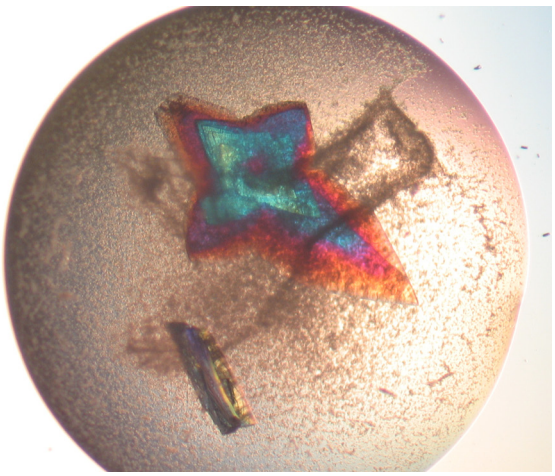


FIGURE 2 | **Crystal of SpIlvC.** Dagger shaped crystals appeared in three weeks at 295 K. Scale bar, 100 μm.

using KARI from Pseudomonas aeruginosa which shows 54% sequence identity (PDB ID: 1NP3; Ahn et al., 2003) as a search model. Preliminary structure refinement using *PHENIX* (Adams et al., 2010) is currently in progress.

METHODS

Macromolecule production

Gene encoding *ilvC* from *Streptococcus pneumoniae* D39 (GenBank accession number: NC_008533.1) was amplified by PCR from genomic DNA. The PCR products were inserted parallel His2 vector (Sheffield et al., 1999) using restriction enzymes *BamHI* and *XhoI* to yield the plasmid

TABLE 1 | Macromolecule production

Source organism	*Streptococcus pneumoniae* D39
DNA source	Genomic DNA
Forward primer	His6-tag, tobacco virus protease site, *BamH*I CGCGGATCCATGACAGTTCAAATGGAATATGAAAAA
Reverse primer	*Xho*I CCGCTCGAGTTATTGATAGATTTTGAATGCATCGTC
Cloning vector	Parallel pHis2
Expression vector	Parallel pHis2
Expression host	*Escherichia coli* BL21(DE3)
Complete amino acid sequence of the construct produced	MTVQMEYEKDVKVAALDGKKIAVIGYGSQGHAHAQNLRDSGRDV IIGVRPGKSFDKAKEDGFDTYTVAEATKLADVIMILAPDEIQQE LYEAEIAPNLEAGNAVGFAHGFNIHFEFIKVPADVDVFMCAPKG PGHLVRRTYEEGFGVPALYAVYQDATGNAKNIAMDWCKGVGAAR VGLLETTYKEETEEDLFGEQAVLCGGLTALIEAGFEVLTEAGYA PELAYFEVLHEMKLIVDLIYEGGFKKMRQSISNTAEYGDYVSGP RVITEQVKENMKAVLADIQNGKFANDFVNDYKAGRPKLTAYREQ AANLEIEKVGAELRKAMPFVGKNDDDAFKIYN

TABLE 2 | Crystallization

Method	Hanging drop vapor diffusion
Plate type	24-well plate
Temperature (K)	at 295K
Protein concentration	11.5 mg ml^{-1}
Buffer composition of protein solution	50 mM Tris-HCl pH 7.5, 150 mM NaCl
Composition of reservoir solution	0.1 M HEPES pH 7.5, 0.1 M NaCl, 1.5 M ammonium sulfate
Volume and ratio of drop	1 µl protein solution with 1 µl reservoir solution
Volume of reservoir	500 µl

pHis2-ilvC encoding His-tagged IlvC. The pHis2-*ilvC* plasmid was transformed into *Escherichia coli* BL21(DE3) strain (Table 1). Cell were incubated at 310K in LB medium supplemented with 50 µg ml^{-1} ampicillin, grown to OD_{600} of 0.6-0.8, then induced with 0.5 mM isopropyl-1-thio-β-D-galactopyranoside at 293K. The induced cells were further grown for 20 h at 293K. Following harvest cells by centrifugation at 4,000 rpm for 15 min at 277 K, the cells were resuspended in buffer A (50 mM Tris-HCl pH 7.5 and 150 mM NaCl), then lysed by sonication. Supernatant containing soluble His-SpIlvC was collected by centrifugation at 13,000 rpm for 1h at 277K and loaded onto a Ni-NTA Agarose resin column (Qiagen).After incubating and washing with buffer B (50 mM Tris-HCl pH 7.5, 0.5 M NaCl and 20 mM imidazole), the protein was eluted in buffer C (50 mM Tris-HCl pH 7.5, 0.5 M NaCl and 0.3 M imidazole). His-tag was cleaved by tobacco etch virus protease (Wu et al., 2009) during dialysis against the buffer A overnight at 277K, and the resulting solution was loaded to Ni-NTA Agarose resin to remove the His-tag and tobacco etch virus protease. Flow-through was collected and further purified on a HiLoad 16/600 Superdex-200 pg (prep-grade) size exclusion column (GE HealthCare) pre-equilibrated with the buffer A. SpIlvC was concentrated using 3 kDa-centrifugal filter (Ambicom Ultra) and measured to 11.5 mg ml^{-1} using Bradford method. Typical protein yield was about 2 mg from 1 l culture. The polydispersity of SpIlvC protein solution in the buffer A was checked by dynamic light scattering on a DynaPro 100 system (Wyatt Technology).

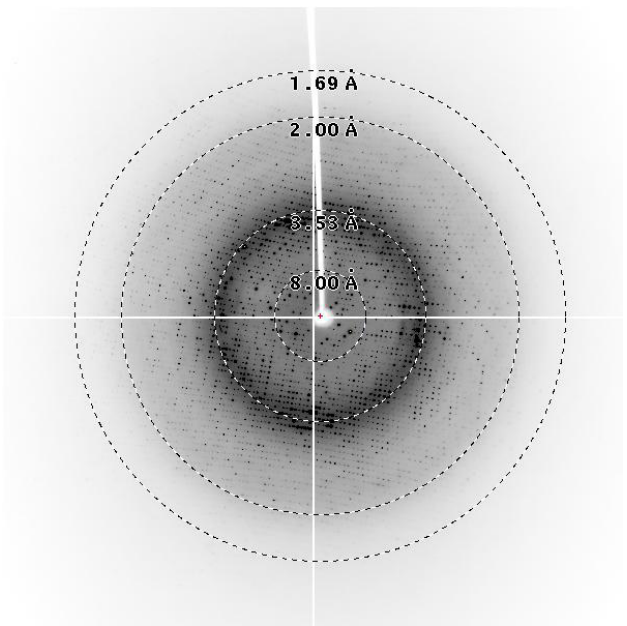


FIGURE 3 | **Representative 1° oscillation X-ray diffraction image from the SpIlvC crystal.** The distance between the crystal and the detector is 160 mm. The diffraction limits are indicated with circles.

Crystallization

TABLE 3 | Data collection and processing statistics

Crystal	SpIlvC
Diffraction source	PAL 7A
Wavelength (Å)	1.0
Temperature (K)	100
Detector	ADSC Quantum Q270
Crystal-detector distance (mm)	160 mm
Total rotation range (°)	1
Exposure time per image (s)	360
Space group	$P2_12_12_1$
a, b, c (Å)	69.1, 104.3, 110.9
α, β, γ (°)	90, 90, 90
Mosaicity (°)	
Resolution range (Å)	41.63 – 1.69 (1.75 – 1.69)[a]
Total No. of reflections	88937 (8739)
No. of unique reflections	88937 (8739)
Completeness (%)	98.98 (98.87)
Redundancy	14.4 (14.6)
$<I/\sigma(I)>$	34.6
$R_{r.i.m.}$[b] (%)	0.178(0.027)
$R_{r.i.m.}$[c] (%)	0.019 (0.126)
Overall B factor from Wilson plot ($Å^2$)	19.92

[a]Values for the outer shell are given in parentheses.

[b] $$R_{r.i.m.} = \sum_h \sum_l \left(\frac{n_h}{n_h - 1}\right)^{1/2} |I_{hl} - < I_h >| / \sum_h \sum_l < I_h >$$

where h refers to a reflection, l to all observations, and n_h the number of observations of reflection h.

[c] $$R_{p.i.m.} = \sum_h \sum_l \left(\frac{1}{n_h - 1}\right)^{1/2} |I_{hl} - < I_h >| / \sum_h \sum_l < I_h >$$

Crystallization was carried out at 295K with 11.5 mg ml^{-1} protein by micro-batch under-oil method using screening kits from Hampton Research and Molecular Dimensions. At 295K, initial SpIlvC crystals were obtained from three screening kits: Screen I, Screen II of Hampton Research and Wizard I of Molecular Dimensions. Initial crystallizing conditions were further optimized by the hanging-drop vapour diffusion method. Diffraction-quality crystal of SpIlvC was obtained by mixing 1 µl protein solution with 1 µl reservoir solution containing 0.1 M HEPES pH 7.5, 0.1 M sodium chloride, and 1.5 M ammonium sulfate at 295K (Table 2).

Data collection and processing

SpIlvC native crystals were transferred to a cryoprotectant solution with 30% glycerol supplemented to the reservoir condition. Diffraction data was collected on single frozen crystal in a gaseous nitrogen stream at 100K over a range 360° with a rotation angle per image of 1.0° at beamlines 5C and 7A at Pohang Accelerator Laboratory. The native SpIlvC crystal diffracted to maximum resolution of 1.69 Å using a crystal-to-detector distance of 160 mm. Data processing and reduction were carried out using *HKL-2000* (Otwinowski & Minor, 1997). The data collection and processing statistics are summarized in Table 3.

ACKNOWLEDGEMENTS

We thank the staff members at Pohang Accelerator Laboratory beamlines 5C and 7A for data collection and Professor Dong-Kwon Rhee at Sungkyunkwan University for providing genomic DNA. This work was supported by the Basic Science Research Program (NRF-2015R1A2A1A15055951) and the Pioneer Research Center Program (2012-0009597) through the National Research Foundation of Korea (NRF) funded by the Ministry of Science, ICT and Future Planning.

AUTHOR INFORMATION

The authors declare no conflicts of interest.

Original Submission: Feb 15, 2017
Revised Version Received: Feb 18, 2017
Accepted: Feb 18, 2017

REFERENCES

Adams, P.D., Afonine, P.V., Bunkóczi, G., Chen, V.B., Davis, I.W., Echols, N., Headd, J.J., Hung, L.W., Kapral, G.J., Grosse-Kunstleve, R.W., McCoy, A.J., Moriarty, N.W., Oeffner, R., Read, R.J., Richardson, D.C., et al. (2010). PHENIX: a comprehensive Python-based system for macromolecular structure solution. *Acta Crystallogr D Biol Crystallogr* **D66**, 213-221.

Ahn, H.J., Eom, S.J., Yoon, H.J., Lee, B.I., Cho, H., and Suh, S.W. (2003). Crystal structure of class I acetohydroxy acid isomeroreductase from Pseudomonas aeruginosa. *J Mol Biol* **328**, 505-515.

Dumas, R., Biou, V., Halgand, F., Douce, R., and Duggleby, R.G. (2001). Enzymology, structure, and dynamics of acetohydroxy acid isomeroreductase. *Acc Chem Res* **34**, 399-408.

Kadioglu, A., Weiser, J.N., Paton, J.C., and Andrew, P.W. (2008). The role of Streptococcus pneumoniae virulence factors in host respiratory colonization and disease. *Nat Rev Microbiol* **6**, 288-301.

Matthews, B.W. (1968). Solvent content of protein crystals. *J Mol Biol* **33**, 491-497.

McCourt, J.A., and Duggleby, R.G. (2006). Acetohydroxyacid synthase and its role in the biosynthetic pathway for branched-chain amino acids. *Amino Acids* **31**, 173-210.

Santiago, B., MacGilvray, M., Faustoferri, R.C., and Quivey, R.G. Jr. (2012). The branched-chain amino acid aminotransferase encoded by ilvE is involved in acid tolerance in Streptococcus mutans. *J Bacteriol* **194**, 2010-2019.

Sheffield, P., Garrard, S., and Derewenda, Z. (1999). Overciming Expression and purification problem of RhoGDI using a family of "Parallel"Expression Vectors. *Protein Expr Purif* **15**, 34-39.

Subashchandrabose, S., LeVeque, R.M., Wagner, T.K., Kirkwood, R.N., Kiupel, M., and Mulks, M.H. (2009). Branched-chain amino acids are required for the survival and virulence of Actinobacillus pleuropneumoniae in swine Infect. *Infect Immun* **77**, 4925-4933.

Wagner, T.K., and Mulks, M.H. (2006). A subset of Actinobacillus pleuropneumoniae in vivo induced promoters respond to branched-chain amino acid limitation. FEMS Immunol *Med Microbiol* **48**, 192-204.

Wong, S.H., Lonhienne, T.G., Winzor, D.J., Schenk, G., and Guddat, L.W. (2012). Bacterial and plant ketol-acid reductoisomerases have different mechanisms of induced fit during the catalytic cycle. *J Mol Biol* **424**, 168-179.