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Expression, purification, crystallization, and X-ray diffraction studies on a PadR-like protein from *Bacillus cereus*

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Transcriptional regulators that belong to the PadR family play a critical role in the regulation of various biological processes, such as detoxification, catabolism, toxin production, and antibiotic resistance. The structural basis of effector and DNA recognition by PadR has recently been provided through our previous structural study. However, it is unclear whether the mechanism is universally used by other PadR family members. As a first step to reveal the transcription regulatory mechanism of *Bacillus cereus* PadR-like protein (bcPLP), we expressed the bcPLP protein in *Escherichia coli* cells and purified it to homogeneity by chromatographic methods. bcPLP was crystallized in PEG 3350 solutions. A bcPLP crystal diffracted X-ray to 1.95Å resolution. Our analysis of the X-ray diffraction data indicates that the crystal belongs to space group $P2_12_12$ and has two bcPLP chains in the asymmetric unit.

INTRODUCTION

Bacteria are directly exposed to various environmental risk factors. As a defensive system against toxic chemicals, bacteria evolved diverse transcription factors that detect danger signals and subsequently regulate gene expression (Grkovic et al., 2002). For example, in *Bacillus subtilis, Bacillus pumilus, Pediococcus pentosaceus*, and *Lactobacillus plantarum*, PadR functions as a negative transcriptional regulator that recognizes toxic phenolic acids and regulates the expression of a phenolic acid-decomposing enzyme (Barthelmebs et al., 2000; Degrassi et al., 1995; Silva et al., 2011; Tran et al., 2008). PadR-like proteins are found in numerous bacteria and form a large family that comprises at least 9,000 members (Finn et al., 2016). In addition to detoxification, PadR family members are involved in the regulation of diverse cellular processes, including virulence factor production, multidrug resistance, antibiotic biosynthesis, and catabolism as observed in *Vibrio cholerae* AphA, *Listeria monocytogenes* LadR, *Streptomyces argillaceus* MtrY, and *Corynebacterium glutamicum* VanR, respectively (De Silva et al., 2005; Florez et al., 2015; Huillet et al., 2006; Morabbi Heravi et al., 2015).

Structural mechanisms used by the *B. subtilis* PadR (bsPadR) transcription factor to bind effector and DNA have recently been revealed (Park et al., 2017a). bsPadR is composed of two domains, an N-terminal domain (NTD) and a C-terminal domain (CTD). bsPadR recognizes the palindromic sequence of operator DNA using the NTD. bsPadR accommodates a phenolic acid effector molecule into a unique interdomain pocket created between the NTD and the CTD. Phenolic acid binding to the interdomain pocket of bsPadR causes the NTD to shift away from the CTD and to dissociate from DNA. However, due to the limited structural study of other PadR family members, it is unclear whether the structural mechanism of bsPadR is applied to the entire PadR family.

Bacillus cereus expresses a PadR-like protein (bcPLP), which exhibits 28% sequence identity with bsPadR. Based on the sequence analysis of bcPLP and bsPadR, the key DNA-binding residues of bsPadR are conserved in bcPLP, suggesting that bcPLP functions as a DNA-binding protein. However, the effector-binding residues of bsPadR are not recapitulated in bcPLP and thus, bcPLP and bsPadR would differ in effector identity or effector-binding mode. As a first step to provide the structural basis of DNA and effector recognition by bcPLP, we have carried out expression, purification, crystallization, and X-ray diffraction studies of the bcPLP protein.

RESULTS AND DISCUSSION

The bcPLP gene was cloned into a modified pET49b vector that was designed to express protein with an N-terminal hexa-histidine tag, and the resulting bcPLP expression plasmid was transformed into *Escherichia coli* BL21 (DE3) cells for protein production (Table 1). The bcPLP protein was overexpressed and then purified by Ni-NTA affinity chromatography and anion exchange chromatography. The final yield of the purified protein was ~15 mg l^{-1}.

The size and purity of the bcPLP protein were analyzed by

TABLE 1 | Protein production information

Source organism	*Bacillus cereus*
DNA source	Genomic DNA
Forward primer*	5'-TTAGGATCCGATGAAGGG AAGAGATGTTGTTTTAGG-3'
Reverse primer*	5'-GTAAGCTTTCATTCTTCTGT TTTATTGTTTTCTTTCGCC-3'
Cloning vector	Modified pET49b
Expression host	*Escherichia coli* BL21 (DE3)
Complete amino acid sequence of the construct produced**	GSAKDPMKGRDVVLGLLMQKELSGYDIK IVFEDVFTHFFDGSFGMIYPTLRQLENEGK IKKEVVMQEGKPNKKMYFITDEGREEFY QYMQTPVEKDVLRSDFLMRMYFGNYSD DVTIKKWIKDEIERKEAYIADLRLKYEKWR VGITFVEEISLDVGIASYSAQVETLKKKLEE LEAKENNKTEE

* Underlined sequence: restriction-enzyme site.
** Underlined sequence: vector-derived residues at the N-terminus.

TABLE 2 | Crystallization condition

Method	Vapour diffusion in sitting drops
Plate type	24-well Cryschem plates (Hampton Research)
Temperature (K)	291
Protein concentration (mg ml^{-1})	10-20
Buffer composition of protein solution	20 mM Hepes, pH 7.4, and 300 mM NaCl
Composition of reservoir solution	20% PEG 3350, 0.1 M sodium citrate, pH 5.6, and 0.2 M ammonium dihydrogen phosphate
Volume of drop	0.5 µl protein solution with 0.5 µl reservoir solution
Volume of reservoir (µl)	500

TABLE 3 | Data collection and processing statistics

Diffraction source	PAL beamline 7A
Wavelength (Å)	0.97939
Temperature (K)	100
Detector	ADSC Quantum 270
Crystal-to-detector distance (mm)	220
Rotation range per image (°)	0.5
Total rotation range (°)	171
Exposure time per image (s)	1
Space group	$P2_12_12$
a, b, c (Å)	60.8 Å, 132.9 Å, 49.3 Å
α, β, γ (°)	90°, 90°, 90°
Resolution range (Å)	30.0-1.95 (2.02-1.95)
Completeness (%)	99.7 (99.8)
R_{merge} (%)	5.6 (46.5)
<I/σ(I)>	48.0 (3.6)
Redundancy	6.7 (6.9)
Total No. of reflections	29,789
No. of reflections	200,837

* Values in parentheses are for the highest resolution shell.

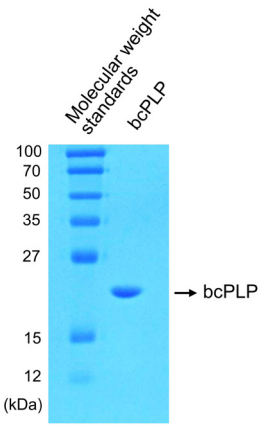

FIGURE 1 | **SDS-PAGE analysis of the bcPLP protein in comparison with molecular weight standards (100 kDa, 70 kDa, 50 kDa, 35 kDa, 27 kDa, 15 kDa, and 12 kDa).**

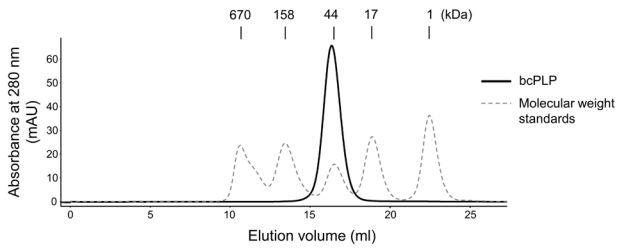

FIGURE 2 | **Gel-filtration chromatography analysis of the bcPLP protein in comparison with molecular weight standards (670 kDa, 158 kDa, 44 kDa, 17 kDa, and 1 kDa).**

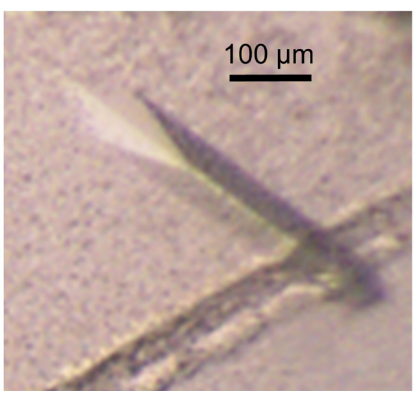

FIGURE 3 | **SeMet-bcPLP crystal (~70×~70×~450 µm) obtained in 20% PEG 3350, 0.1 M sodium citrate, pH 5.6, and 0.2 M ammonium dihydrogen phosphate.**

SDS-PAGE (Figure 1). The bcPLP protein migrated as a single band between the 15 kDa and 27 kDa protein standards to be consistent with its calculated molecular weight (21.8 kDa). The finally purified bcPLP protein showed at least 95% purity. The oligomeric state of the bcPLP protein was determined by gel-filtration chromatography (Figure 2). The bcPLP protein was

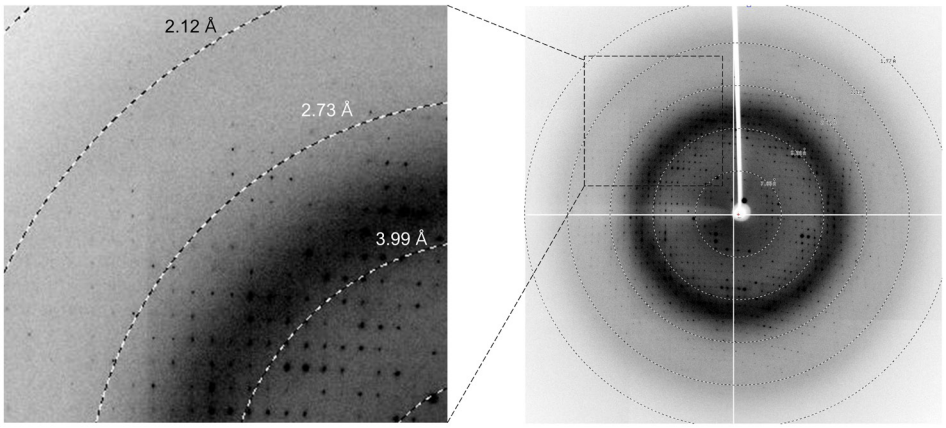

FIGURE 4 | Representative X-ray diffraction image of the SeMet-bcPLP crystal. Resolution circles are shown.

eluted at an elution volume of 16.4 ml, which corresponds to an apparent molecular weight of ~41 kDa based on the relationship between the elution volumes of molecular weight standards and their molecular weights, indicating that bcPLP is dimeric in solution.

bcPLP formed rod-shaped crystals (~70×~70×~450 μm) in PEG 3350 solutions (Figure 3 and Table 2). We have collected X-ray diffraction data to 1.95 Å resolution using a single crystal of selenomethionine-incorporated bcPLP (SeMet-bcPLP) (Figure 4). A summary of data collection and processing statistics is shown in Table 3. The bcPLP crystal belonged to the orthorhombic space group $P2_12_12$ with unit-cell parameters of a = 60.8 Å, b = 132.9 Å, c = 49.3 Å. 200,837 observed reflections were merged to produce 29,789 unique reflections with a merging R factor of 5.6% and a completeness value of 99.7%. Solvent content and Matthews coefficient analysis suggests that the asymmetric unit of the crystal contains two copies of bcPLP (solvent content, 46.2%; Matthews coefficient Vm, 2.29 $Å^3$ Da^{-1}) (Kantardjieff and Rupp, 2003; Matthews, 1968). Structure determination by single-wavelength anomalous diffraction is currently underway.

METHODS

Cloning

The gene that encodes the full-length protein of bcPLP (GenBank accession number, NP_831531; residues 1-179) was amplified by PCR from the genomic DNA of *B. cereus* using forward (5'-TTA**GGATCC**GATGAAGG GAAGAGATGTTGTTTTAGG-3', *Bam*HI restriction-enzyme site in bold) and reverse (5'-GT**AAGCTT**TCATTCTTCTGTTTTATTGTTTTCTTTCGCC-3', *Hind*III restriction-enzyme site in bold) primers. The PCR product was digested using *Bam*HI and *Hind*III enzymes and ligated into a modified pET49b vector that was designed to express protein in N-terminal fusion with a hexa-histidine tag and a thrombin cleavage site (Song et al., 2017). The ligation product was transformed into *E. coli* strain DH5α, and a correct clone was selected based on restriction-enzyme digestion and DNA sequencing.

Expression and purification

The bcPLP expression vector DNA was transformed into *E. coli* strain BL21 (DE3) for overexpression. Cells were grown at 37°C in LB medium containing 50 μl ml^{-1} kanamycin. When the optical density of the culture at 600 nm reached ~0.6, isopropyl β-D-1-thiogalactopyranoside was added to a final concentration of 1 mM into the culture to induce the overexpression of the bcPLP protein. The cells were further grown for ~17 hr at 18°C, harvested by 20-min centrifugation, and lysed by sonication in 50 mM Tris, pH 8.0, 200 mM NaCl, and 1 mM phenylmethylsulfonyl fluoride. The cell lysate was cleared by centrifugation. The resultant supernatant was incubated with Ni-NTA resin (Qiagen) in the presence of 10 mM imidazole at 4°C for 2 hr. The resin was applied onto an Econo-Column (Bio-Rad) and washed using 10 mM imidazole, 50 mM Tris, pH 8.0, and 200 mM NaCl. The bcPLP protein was eluted using 250 mM imidazole, 50 mM Tris, pH 8.0, and 200 mM NaCl. The eluted bcPLP protein was dialyzed against 20 mM Hepes, pH 7.4, and then digested by thrombin at 18°C for 3 hr to cleave the hexa-histidine tag off. The tag-free bcPLP protein was further purified by anion-exchange chromatography using a Mono Q 10/100 column. The column was equilibrated with 20 mM Hepes, pH 7.4. The bcPLP protein was eluted using a 0.0-0.5 M NaCl gradient. The SeMet-bcPLP protein was produced in the presence of L-selenomethionine using *E. coli* strain B834 (DE3) and purified in a manner identical to the native bcPLP protein. The purified bcPLP was concentrated to 10-20 mg ml^{-1} for crystallization.

Gel-filtration chromatography

To determine the oligomeric state of the bcPLP protein, gel-filtration chromatography was performed. A Superdex 200 10/300 column (GE Healthcare) was equilibrated with running buffer containing 20 mM Hepes, pH 7.4, and 150 mM NaCl. The bcPLP protein or molecular weight standards (Bio-Rad) were injected into the column in a volume of 300 μl, and the running buffer was flowed through the column. Protein elution was monitored by measuring absorbance at 280 nm.

Crystallization

bcPLP crystallization was carried out by the sitting-drop vapour-diffusion method. 0.5 μl of 10-20 mg ml^{-1} bcPLP protein solution was mixed with 0.5 μl of reservoir solution in 96-well crystallization plates (Molecular Dimensions) for initial crystallization screening or in 24-well Cryschem plates (Hampton Research) for crystallization optimization. The mixed drop was equilibrated against reservoir solution at 18°C. Diffractable SeMet-bcPLP crystals were obtained in 20% PEG 3350, 0.1 M sodium citrate, pH 5.6, and 0.2 M ammonium dihydrogen phosphate.

X-ray diffraction

A single SeMet-bcPLP crystal was transferred from a crystallization drop into a cryoprotection solution containing 25% ethylene glycol, mounted on a goniometer, and flash-cooled under a cryo-steam at -173°C. A total of 342 X-ray diffraction images were collected with a 0.5° oscillation by an exposure time of 1 s per frame using an ADSC Quantum 270 detector in beamline 7A of the Pohang Accelerator Laboratory, Republic of Korea (Park et al., 2017b). Diffraction data were indexed, integrated, and scaled using the HKL2000 package (Otwinowski and Minor, 1997).

CONFLICT OF INTEREST

The authors declare no potential conflicts of interest.

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