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Short crystallization paper

Crystallization and preliminary crystallographic analysis of *Bacillus thuringiensis* AHL-lactonase

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Abstract

The quorum sensing (QS) systems in Gram-negative bacteria are mostly associated with diffusible *N*-acyl-L-homoserine lactones (AHLs). AHL-degrading enzymes hydrolyze the AHLs into inactive molecules, thereby blocking the QS systems that are closely linked to virulence factor production and biofilm formation. Consequently, these enzymes have recently attracted intense interest for the development of anti-infection therapies for plants and animals. However, despite significant progress in the investigation of AHL-degrading enzymes, no structure is yet available. Accordingly, this study reports on the expression and purification of the AHL-lactonase from *Bacillus thuringiensis* subsp. *kurstaki* HD263, as well as the successful crystallization of the enzyme. High-quality native crystals were obtained and a complete data set collected at 2.0 Å resolution. The native crystal was found to belong to the space group $P2_12_12_1$, with unit cell parameters $a=52.7$ Å, $b=55.9$ Å, and $c=74.1$ Å and one molecule in the asymmetric unit. MAD data were also collected at 2.4 Å resolution for a SeMet-substituted crystal.

Keywords: Quorum sensing; AHL-lactonase; *N*-acyl-L-homoserine lactone; Crystallization

Quorum sensing (QS) systems are widespread in bacterial species and have attracted interest, as they facilitate cells within bacterial communities to communicate with each other, resulting in the regulation of diverse phenotypes [1]. The QS mechanisms in bacteria are mostly based on the production and sensing of small chemicals. In Gram-negative bacteria, the most intensively studied chemical signals are *N*-acyl-L-homoserine lactones (AHLs), which are synthesized by an AHL synthase, usually a member of the LuxI family, and can freely diffuse out of and into the bacterial cell. Subsequently, an AHL receptor protein belonging to the LuxR family of transcriptional regulators regulates various processes of target gene expression when the AHL production reaches a critical threshold concentration in a cell density-dependant manner [2]. Bacterial cell-to-cell communication via the diffusible AHLs regulates the expression of various phenotypes, such as biofilm formation and virulence factor production, resulting in troubles in a wide spectrum of plants and animals [3,4].

The disintegration of such AHL-mediated QS systems has thus been targeted as a promising approach for a novel control technique of virulence gene expression in bacterial infections. A 250-residue-long AHL-lactonase coded by the *aiiA* gene, which was first identified in a *Bacillus* sp., has been employed to block QS by hydrolyzing the lactone ring of AHLs into inactive chemicals [5]. In addition, a number of *Bacillus* species with AHL-inactivating properties have

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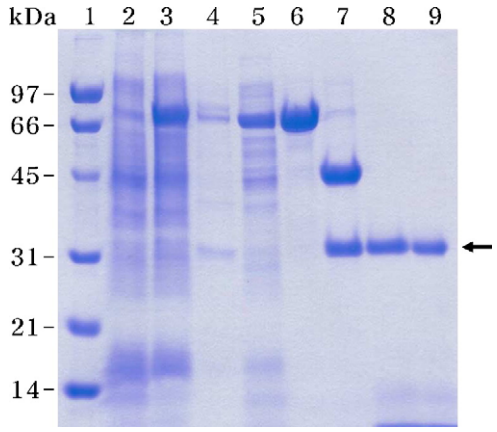

Fig. 1. SDS-PAGE analysis of expression and purification of the AHL-lactonase. Lane 1, protein size markers; lane 2, uninduced *E. coli* XL1-Blue cell lysate; lane 3, induced cell lysate; lane 4, insoluble fraction; lane 5, soluble fraction; lane 6, eluted MBP-6×His-tagged AHL-lactonase; lane 7, rTEV-treated MBP-6×His-tagged AHL-lactonase; lane 8, purified native AHL-lactonase; lane 9, purified SeMet-substituted AHL-lactonase.

recently been identified and some have been found to possess homologues of *aiiA* showing high sequence similarities (above 95%) [6]. Lee et al. reported on the distribution of *aiiA*-homologous genes in the insecticidal *Bacillus thuringiensis* subspecies and demonstrated the possibility of the biological control of plant-pathogenic bacteria [6]. AHL-acylases for inactivating QS signals have also been detected in *Ralstonia* sp. and *Variovorax paradoxus*. In contrast to AHL-lactonases, these enzymes hydrolyze the amide bond connecting the homoserine lactone ring to the acyl chain, releasing homoserine lactone and fatty acid [7]. AHL-degrading enzymes, when expressed either in transgenic plants or bacterial pathogens, have also been found to block bacterial quorum sensing and prevent bacterial population density-dependent infection [7,8,9]. One of the most interesting aspects of AHL-lactonases is that the enzymes contain a conserved sequence ^{104}HXHXDH^{109}–H^{169} [10], which is identical to the Zn^{2+}-binding motif of several metallohydrolases, including the glyoxylase II and arylsufatase enzyme families, and β-lactamases [11]. Wang et al. proposed that the ^{106}HXDH^{109}–H^{169} of AHL-lactonases represents a novel catalytic motif, which does not rely on zinc or other metal ions for activity. However, the mechanism that rules out the possibility of metal involvement in the enzyme catalysis remains far from clear.

Accordingly, for a structural analysis to better understand the catalytic mechanism and biological role of QS-quenching AHL-lactonases, the current study reports on the purification of the AHL-lactonase from *B. thuringiensis* subsp. *kurstaki* HD263, along with the first successful crystallization and preliminary X-ray study of an AHL-lactonase.

The *aiiA* gene coding for the AHL-lactonase was amplified from the chromosomal DNA of *B. thuringiensis* subsp. *kurstaki* HD263 [6] by a polymerase chain reaction (PCR) using the following primers: 5′-CATGCCATG-GATATGACAGTAAA-3′ and 5′-AACTGCAGCTATAT-ATATTCCGG-3′, which carried the *Nco*I and *Pst*I restriction sites. The PCR product was then gel purified (Qiagen) and the *aiiA* gene subcloned into the *Nco*I and *Pst*I sites of the pMal-His-Parallel1 vector, kindly supplied by Dr. Zygmunt S. Derewenda at the University of Virginia, a maltose-binding protein (MBP) and hexahistidine-fusion protein expression vector containing a recombinant TEV protease (rTEV) cleavage site. The integrity of the insert was verified by direct DNA sequencing. *E. coli* XL1-Blue (Stratagene) was transformed with the expression plasmid and selected on LB agar plates containing ampicillin. *E. coli* XL1-Blue cells harboring the plasmid were then grown in an LB medium (2 l) containing ampicillin at 37 °C until reaching an OD_{600} between 0.6 and 0.8. After being cooled to 25 °C, the cells were induced with 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG) and grown for a further 14 h. The cells (~10 g) were then harvested by centrifugation at $5000 \times g$ for 20 min at 4 °C. The cell pellets were resuspended in 100 ml of ice-cold buffer A (50 mM Tris–HCl, pH 8.0, and 300 mM NaCl) and disrupted by ultrasonication. The crude cell extracts were centrifuged at

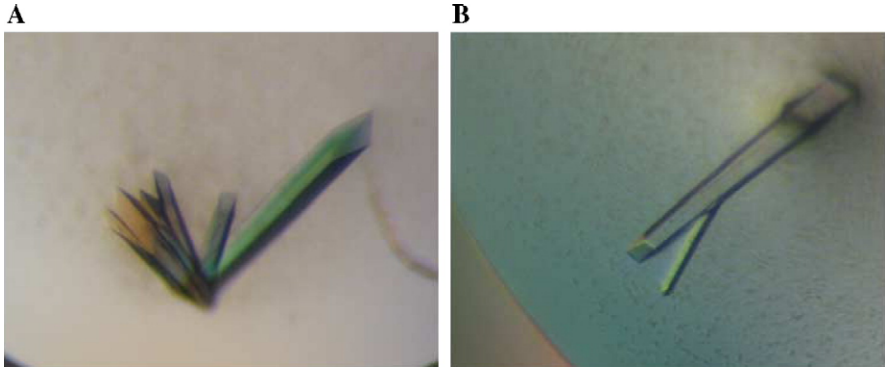

Fig. 2. Native (A) and SeMet-substituted (B) AHL-lactonase crystals. The typical dimensions of the diffraction-quality native and SeMet-substituted crystals were approximately $0.15 \times 0.15 \times 0.5$ mm and $0.075 \times 0.075 \times 0.3$ mm, respectively.

11,000 × *g* for 1 h at 4 °C. The cell lysate containing the MBP-6×His-tagged AHL-lactonase was then bound to 10 ml of Ni-NTA agarose (QIAGEN) equilibrated with buffer A for 2 h at 4 °C. After the resin was washed with 1 l of buffer A, the bound proteins were eluted in 50 ml of buffer A containing 200 mM imidazole. The MBP-6×His-tag was then released from the AHL-lactonase by incubating with rTEV protease (GIBCO), followed by Ni-NTA agarose chromatography and size exclusion chromatography. After purification, the proteins had the two-residue cloning artifact (Met-Asp) at their N-termini. The final protein yield was approximately 0.75 mg/l, as determined by the Bradford procedure (Bio-Rad Protein Assay). The homogeneity of the protein was assessed by 10% SDS-PAGE and Coomassie Blue staining (Fig. 1). The purified protein was then dialyzed against 20 mM Tris–HCl, pH 7.5, concentrated to ~7 mg/ml using a YM-10 membrane (Amicon), and stored at −80 °C for use in the crystallization trials. The SeMet-substituted protein was expressed in the methionine auxotroph strain *E. coli* B834 (DE3) (Novagen) in a minimal medium (2 l) supplemented with 50 mg/ml SeMet under the same conditions as the native. The purification of the SeMet substituted protein was identical to that of the native protein, except for the addition of 5 mM methionine to all the buffers. The final amount of the purified protein was approximately 1 mg.

A Crystal Screen (Hampton Research) was used for the preliminary screening with the native protein. Subsequently, the crystallization conditions were optimized around 0.1 M Tris–HCl, pH 8.0, containing 30% PEG 4000 and 0.2 M $MgCl_2$. The sitting-drop vapor-diffusion method was used for all the crystallization trials. One microliter of 7 mg/ml protein and 1 μl of the reservoir solution were mixed and equilibrated against the reservoir solution and stored at 21 °C. Crystals appeared within 2 h and grew for about 5 days to reach their maximal dimensions of approximately 0.15 × 0.15 × 0.5 mm (Fig. 2A). SeMet substituted protein crystals were also obtained under the same crystallization conditions with 5 mM methionine using the sitting-drop vapor-diffusion technique at 21 °C. Crystals appeared with maximal dimensions of 0.075 × 0.075 × 0.3 mm after 5 days of growth (Fig. 2B).

The crystals were transferred to a cryo-protecting solution containing 35% PEG 4000, 0.1 M Tris–HCl, pH 8.0, 0.2 M $MgCl_2$, and 10% glycerol in the same well where they were grown. First, 10 μl of the mother liquor was added to the well, then 5 μl aliquot of the cryo-protecting solution was gradually added to the drop and the same amount removed from the drop after mixing. After the complete exchange of the mother liquor with the cryo-protecting solution, the crystals were fished and placed immediately in a −173 °C nitrogen–gas stream. The native crystal was then rotated a total of 180° (1° rotation) at a wavelength of 1.12710 Å. In addition, a three-wavelength MAD data set of the SeMet-labeled crystal based on 180 images (1° rotation) was collected at 0.97940 Å (edge wavelength), 0.96836 Å (remote wavelength), and 0.97928 Å (peak wavelength). All the data were collected at beam line 6B of the Pohang Accelerator Laboratory (PAL), Pohang, Korea, using a Bruker AXS Proteum300 CCD detector (Madison, WI, USA). The data were then indexed, integrated, and scaled using the HKL2000 suite [12]. The native crystal was found to belong to space group $P2_12_12_1$, with unit cell parameters a = 52.7 Å, b = 55.9 Å, and c = 74.1 Å. The native data set was 97.7% complete at a 2.0 Å resolution, the asymmetric unit contained one molecule, and the value of the Matthews constant V_M [13] was 1.9 Å^3/Da, corresponding to a solvent content of 35.2%. The SeMet-substituted crystal was also determined to be $P2_12_12_1$, with unit cell parameters a = 52.7 Å, b = 55.9 Å, and c = 73.7 Å, where the asymmetric unit contained one molecule and the V_M was 1.9 Å^3/Da, corresponding to a solvent content of 34.8%. The data collection statistics are summarized in Table 1. Se sites were determined using the program SOLVE [14] based on 2.4 Å anomalous data. Three Se atoms were identified and the details will be reported elsewhere.

Table 1
X-ray data collection statistics

	Native	SeMet		
		Edge	Peak	Remote
Wavelength (Å)	1.12710	0.97940	0.97928	0.96836
Resolution range (Å)	2.0 (2.07–2.00)	2.4 (2.49–2.40)	2.4 (2.49–2.40)	2.4 (2.49–2.40)
Space group	$P2_12_12_1$	$P2_12_12_1$		
Unit cell parameters	a = 52.7 Å, b = 55.9 Å, c = 74.1 Å	a = 52.7 Å, b = 55.9 Å, c = 73.7 Å		
Total reflections	104,991	49,525	59,426	49,774
Unique reflections	15,232	8533	8767	8371
Completeness (%)	99.0 (97.7)	98.4 (95.7)	99.7 (99.8)	98.0 (88.7)
R_{merge} (%)[a]	10.5 (41.3)	8.2 (41.8)	9.0 (32.2)	10.4 (52.2)
Average $I/\sigma(I)$	18.4 (4.4)	24.8 (3.8)	33.7 (6.6)	17.6 (2.3)

Values in parentheses refer to the highest resolution shell.
[a] $R_{merge} = \Sigma|I_i - \langle I \rangle| / \Sigma I$, where I_i is the intensity of the ith observation and $\langle I \rangle$ is the mean intensity of the reflections.

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