A THESIS FOR THE DEGREE OF MASTER OF SCIENCE

Structural analysis of HlyU from *Vibrio vulnificus*, a toxin-inducing transcriptional activator

비브리오 패혈증균 독성 발현 전사 인자 HlyU의 구조적 분석

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석사학위논문

Structural analysis of HlyU from *Vibrio vulnificus*, a toxin-inducing transcriptional activator

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이 논문을 석사학위논문으로 제출함

2017년 8월

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2017년 8월

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Abstract

Vibrio vulnificus is a foodborne pathogenic bacterium that is present in marine environments. The infection results from uptake of contaminated food or direct contacts on the injured skin, causing severe gastroenteritis or fatal septicemia in a mortality rate of more than 50% within a couple of days. The most important virulence factor of *V. vulnificus* is a RtxA toxin that can kill the host cells by forming pores on the membrane. Induction of RtxA toxin is mediated by a transcriptional regulator HlyU, which belongs to ArsR/SmtB protein family that operates as a homodimer. To date, the crystal structures of HlyU from *Vibrio cholera* and *V. vulnificus* have been determined. However, it remains elucidated how HlyU is activated and is participated in transcription of *rtxA* at a molecular level. To reveal the mechanism of action of HlyU in *V. vulnificus*, I first determined a minimal DNA sequence for binding of HlyU, indicating that at least two units of the HlyU dimer is bound to the promoter region of rtxA1. Subsequently, the structural determination of the complex of DNA-*Vv*HlyU was tried by x-ray crystallography. The crystals of complex of HlyU and the DNA fragment were obtained, and the optimization of the crystallization conditions is now in progress to determine the crystal structure.

Recently, a research group in this department discovered a synthetic chemical compound called CM2660 inhibits the function of HlyU when the compound was treated to the bacteria. In order to identify mechanism of how CM2660 inhibits the function of HlyU, I determined crystal structure of HlyU from *V. vulnificus* with a protein sample treated with CM2660. A significant change of the inter-subunit orientation by the compound was found with the reduced interval of the two DNA binding α-helices in the HlyU dimer. This conformational change in the dimer is expected to be associated with a lower binding ability to DNA, leading to inactivation of the HlyU function. Although the compound itself was not observed in the crystal structure, the distance between two cysteine, Cys30 and Cys96 in each subunit, was shortened when compared to the compound-free structure previously reported. In addition, I further identified that CM2660 did not disrupt the dimeric form of HlyU and the presence of extra electron density map between Cys30 and Cys96. Taken together, it is expected that CM2660 may promote the interaction or modification at Cys30 and consequently result in conformational change in the dimer. In order to analyze modification of the cysteine residues, mass spectrometric analysis is under way. This study contributes to the understanding of the HlyU molecular mechanism, which is directly related to the pathogenicity of the fatal foodborne bacterium *V. vulnificus*.

***Keywords*: *Vibrio vulnificus*, transcriptional activator, HlyU, putative inhibitor, crystal structure**

***Student Number:* 2015-23133**

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Ⅰ. Introduction

Vibrio vulnificus is an aerobic gram-negative bacterium, which is highly pathogenic to human. It commonly lives in marine environments and in the human body [1, 2]. *V. vulnificus* is highly cytotoxic to eukaryotic cells, and this cytotoxicity is generally regarded as the hallmark of the pathogenicity of this microorganism [3]. Exposure to contaminated water or ingestion of contaminated food or direct administration of the bacteria to injured skin can cause severe gastroenteritis or fatal septicemia with a rapid progress, resulting in a mortality rate of more than 50% within a couple of days [4].

There are two major cytotoxins identified in this organism: cytolysin/hemolysin (encoded by *vvhA*) and RTX (repeats in toxin; encoded by *rtxA1*). The RTX toxin forms pores on cell membranes only after the contact of the bacterium with the host cell. This action of the toxin is required for *V. vulnificus* virulence in mice by promoting bacterial colonization at the infection site and subsequent invasion into the bloodstream [5]. Moreover, the decreased cytotoxic activity and the virulence due to a mutation in the *rtxA* gene were observed in other research, implicating its critical role in the cytotoxic activity and virulence of *V. vulnificus* [4]. Recently, Liu et al. reported that activation of the *rtxA* gene expression is controlled by HlyU at the transcription initiation level [6]. Furthermore, the transcription of the RTX

operon was decreased in a *hlyU* mutant through transcriptome analysis using DNA chip [3]. The activity of HlyU has been confirmed by in vivo-induced antigen technology and proved that HlyU is essential for *V. vulnificus* virulence in mice [5]. HlyU binds to the promoter region which is preoccupied by H-NS, a global repressor in many bacteria, leading to depression of the repression function of H-NS [7]. Accordingly, HlyU has been implicated as an important transcriptional activator of the virulence factors by de-repressing the function of H-NS factors and common in *Vibrio* species [8-10].

The transcriptional activator HlyU belongs to ArsR/SmtB protein family, which is small regulatory proteins containing a helix-turn-helix motif [8]. Most ArsR/SmtB protein family members has a metal binding site, which is important in turning on the transcriptional activity by capturing the metal ion in it. Dissimilar to ArsR/SmtB protein family, HlyU is likely not to have the corresponding metal binding since the residues lining the metal binding site are replaced in HlyU. Therefore, it's a plausible explanation that HlyU might not be involved in the metal binding for its function.

To date, the crystal structures of HlyU from *Vibrio vulnificus* and *Vibrio cholerae* have been determined [11]. HlyU from *V. vulnificus* (*Vv*HlyU) shares 78% sequence identity with HlyU from *V. cholerae* (*Vc*HlyU), exhibiting similar secondary structure elements. In the details of structure of *Vc*HlyU, the Cys38 of *Vc*HlyU and its equivalent residue Cys30 of *Vv*HlyU were noted. The *Vc*HlyU structures

represented two modification states at Cys38. In the presence of a reducing agent tris(2-carboxyethyl) phosphine (TCEP), the extra electron density map near Cys38 wasn't observed in *Vc*HlyU structure. Remarkably, an electron density map close to the sulfur atom of the cysteine residue was found and considered as modification of cysteine to CSO, in an oxidized form of *Vc*HlyU in the absence of reducing agent [11]. Therefore, it seemed that Cys38 of *Vc*HlyU is sensitive to oxidative stress, and its oxidation is limited in oxygen-limiting environments, such as intestine in human. The sensitive oxidation of Cys38 suggests a potential of HlyU as thiol-based redox switches. Thus, this finding would be applicable to the *Vv*HlyU.

According to the crystal structures of *Vv*HlyU and *Vc*HlyU, important residues playing role in making a dimeric interface or secondary structure elements for the binding were observed. Especially, among those residues, the importance of cysteine residues was emphasized. The deduction in distance between two cysteine which is presented on dimeric interface in both of *Vc*HlyU and *Vv*HlyU (Cys38 and Cys104 for the *V. cholerae* and Cys30 and Cys96 for the *V. vulnificus*) caused structural modification on the wing and the recognition α helix contacting DNA and resulted in DNA-unbound form. It is in consistency with result of MD simulation from *V. vulnificus [12]*.

A previous research has identified that HlyU binds directly to a region upstream of the operon where rtxA1 is located [5]. During the preparation of the experiment, I had doubt about the size of binding sequence which is too long for the HlyU

dimeric unit to cover. I speculated that at least two HlyU dimeric units might be bound to the promoter region sequentially to cover the long DNA sequence [4, 13].

In this study, I found the minimum binding unit of a HlyU dimer and also determined the structure of *Vv*HlyU incubated with a chemical compound, N- (4- oxo- 4H- thieno[3,4- c]chromen- 3- yl)- 3- phenylprop- 2- ynamide, or shortly CM2660, as a putative inhibitor. This structure further was analyzed with mass focusing on cysteine and its neighboring residues regarded as major components causing conformational changes.

Ⅱ. Materials and Methods

2.1. Plasmid construction

The template DNA of the HlyU from *V. vulnificus* was kindly provided by Dr. Jeong-Sun Kim at Cheonnam University [4]. The recombinant plasmid (pProEx-HTc-*Vv*HlyU) was transformed into *Escherichia coli* strain C43(DE3) [14] using heat-shock method and incubated overnight at 37°C for protein production.

2.2. DNA fragment

To define the minimal binding unit, I divided into two fragments of DNA from the binding sequence discovered by Liu *et al*. The two fragments were then oligo synthesized (table. 1). These each forward and reverse DNA fragments were annealed by decreasing temperature slowly from 45°C results in dsDNA, suitable form for DNA-protein complex. The modified fragment Ⅰ was used for crystallization.

2.3. Purification of *Vv*HlyU

2.3.1. Overexpression

The recombinant HlyU was inoculated by picking a single colony from *E.coli* strain

into 25ml of LB medium with 50μg/ml ampicillin (Duchefa, Netherlands) and incubated at 37°C overnight. The *E. coli* strain was cultured in 2.0 L of LB medium including appropriate antibiotics until an OD_{600} of 0.8 and protein production was induced with 0.5 mM IPTG at 30℃. Cells were harvested 6 h after induction, and the cell pellet was resuspended with 50 ml of lysis buffer containing 20 mM Tris-HCl (pH 7.5), 500 mM NaCl, and 2 mM 2-mercaptoethanol. After homogenization by Sonicator, the cell lysate was acquired by centrifugation at 13,000 rpm for 30 min.

2.3.2. Affinity chromatography

The 6X His-tagged HlyU protein of the cell lysate was bound to Ni^{2+}-NTA resin by rolling for 1 h at 4°C. The Ni^{2+}-NTA resin bound with recombinant HlyU washed out with buffer containing 500 mM NaCl, 20 mM Tris-HCl (pH 7.5), 40 mM Imidazole (pH 7.5), and 2 mM β-mercaptoethanol to remove the other proteins. Elution was carried out by using lysis buffer containing 250 mM Imidazole (pH 7.5). The size and purity of eluted protein was identified by SDS-PAGE.

2.3.3. Size exclusion chromatography (SEC)

The eluted proteins were further purified by size exclusion chromatography (HiLoad Superdex 200 26/600; GE Healthcare). The column was pre-equilibrated with buffer containing 300 mM NaCl, 20 mM Tris-HCl (pH 7.5), and 2 mM β-

mercaptoethanol. The fractions corresponding to HlyU were concentrated up to 9 mg/ml using a centrifugal filter concentration device (Millipore, Temecula, CA; 10 kDa cutoff). The purity of protein was confirmed by SDS-PAGE. After the concentration, the proteins were incubated with chemical compound, putative inhibitor, by 1:10 molecular ratio for 30 min on ice. They were further centrifuged at 13,000 rpm for 7 min to remove the pellet made during the incubation if it was necessary. The final incubated sample was stored frozen at -80℃.

Also each of the binding DNA fragment Ⅰ/Ⅱ were incubated with proteins by 1: 1.2 to 1.5 mole ratio for 30 min at 4°C. Before the incubation, 0.1mM final concentration of divalent ions such as magnesium chloride were used to protein for an hour at 4°C to assist making stabilized dsDNA complex form. The mixture applied to column again for separating bound form from unbound form. The fractions identified as bound form were centrifuged and stored under -80℃.

2.4. Crystallization

Crystallization of HlyU incubated with CM2660 and DNA-HlyU complex were performed using the vapor-diffusion hanging drop method at 14℃ under a mother liquor containing 0.1 M Tris-HCl (pH 8.5), 8% (*wt*/*vol*) PEG 8K for the DNA-HlyU complex and 0.1M HEPES (pH 8), 20% (*wt*/*vol*) PEG 4K, and 10% (*vol*/*vol*) 2-propanol for CM2660-incubated HlyU. The compound incubated crystals were

flash-frozen using 20% (*vol*/*vol*) sorbitol as a cryoprotectant in a nitrogen stream at -173℃ prior to collecting the X-ray diffraction dataset with the Pohang Accelerator Laboratory beamline 5C and were processed with the HKL2000 package [15].

2.5. Structural determination and refinement

The crystals of the compound-incubated *Vv*HlyU belonged to the spacegroup $P4_12_12$, with unit cell dimensions of a = 35.1 Å , b = 35.1 Å , and c = 180.4 Å (Table 2). The structure was determined using the MOLREP program in the CCP4 package by the molecular replacement method and a search model taken from the HlyU from *V. vulnificus* CMCP6 (PDB code: 3JTH)[4]. The final structure of HlyU incubated with compound was refined at a 2.1 Å resolution with an R factor of 0.23% and an R_{free} of 0.29% using the PHENIX program [16]. Further details on the structure determination and refinement are given in Table 2.

2.6. Analytic size exclusion chromatography

To find out whether the compound is responsible for disrupting dimerization of HlyU or not, size exclusion chromatography using a HiLoad Superdex GL 200 26/600 column (GE Healthcare). The column was pre-calibrated with following size standard proteins: ferritin (440 kDa), aldolase (158 kDa), conalbumin (75 kDa), ovalbumin (44 kDa), carbonic anhydrase (29 kDa), and RNase A (13.7 kDa). The

samples treated with the compound or not were then applied to the column and eluted fractions at the peak were electrophoresed to confirm the molecular size of HlyU.

2.7. Identification of DNA-protein complex

The proteins incubated with dsDNA fragments by 1:1.5 molar ratio for 30 min at 4℃ were eluted from size exclusion chromatography as described above. The fraction considered as complex were measured spectrophotometrically using spectrophotometer in a range from 200 nm to 300 nm.

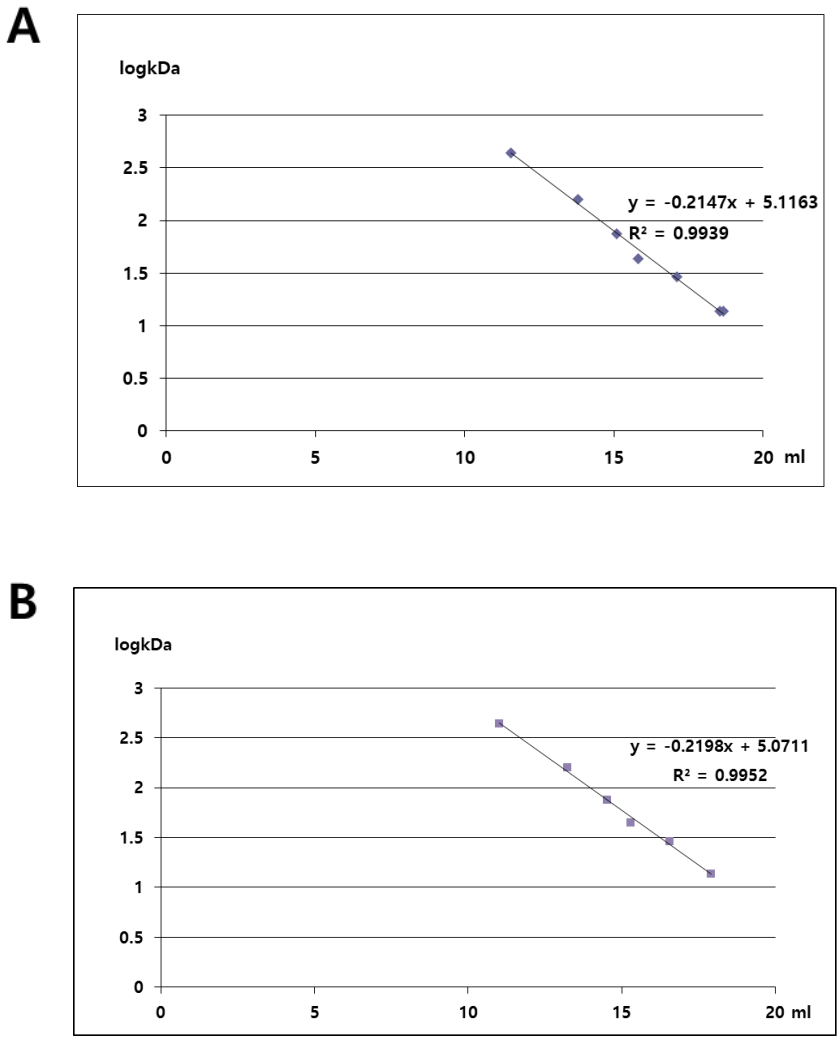


Figure 1. Standard curve of analytic size exclusion chromatography

(A) Used in experiment of *Vv*HlyU-DNA, (B) Used in experiment of *Vv*HlyU-CM2660. Analytic size exclusion chromatography was calibrated based on the following standard proteins represented in triangle: ferritin (440 kDa), aldolase (158 kDa), conalbumin (75 kDa), ovalbumin (44 kDa), carbonic anhydrase (29 kDa), and RNase A (13.7 kDa).

Ⅲ. Results

3.1. Overexpression and purification of *Vv*HlyU

The crystal structure of *V. vulnificus* HlyU was previously determined [4]. However, the structure of the DNA-protein complex and the inhibitor-protein complex has not been characterized yet. Fortunately, the expression vector for *Vv*HlyU was kindly provided by Dr. Jeong-Sun Kim at Cheonnam University, and the expression level was sufficient for the following studies. The recombinant protein was successfully produced in the *E. coli* expression system. The HlyU, 6X His-tagged protein at N-terminus, was purified by affinity chromatography. It was further purified by size exclusion chromatography. The SDS-PAGE was carried out at each step to verify the purities of the samples (Fig. 2).

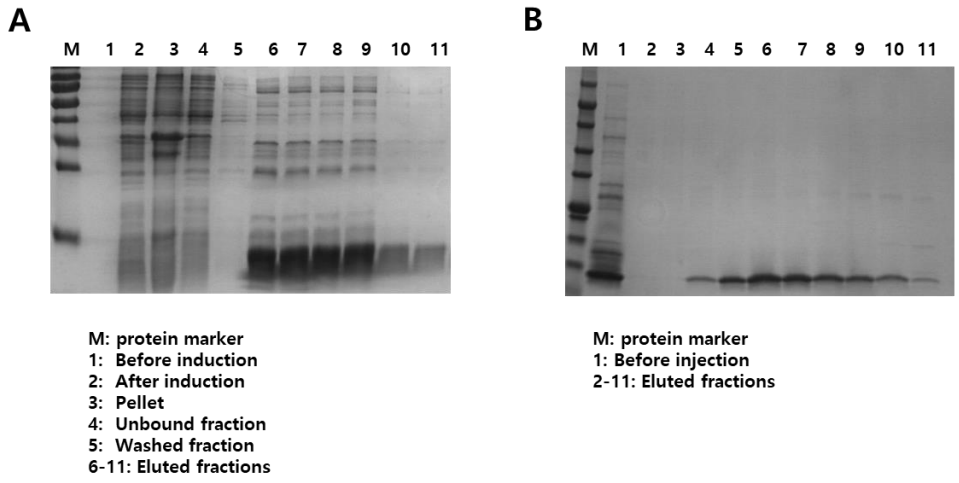


Figure 2. Elution profile from Ni-NTA affinity chromatography (A) and Size exclusion chromatography (B).

3.2. Minimal unit of DNA for binding to *Vv*HlyU

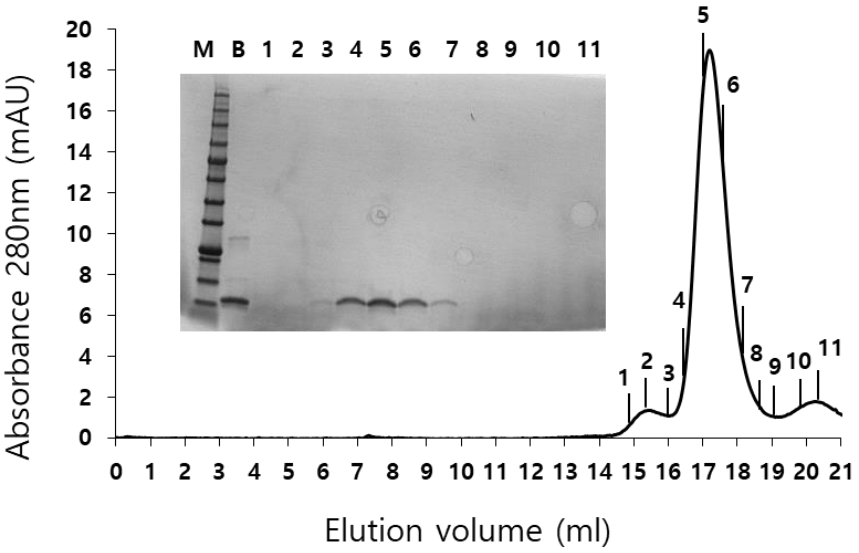
Binding site of *Vv*HlyU at promoter region was previously revealed by another group [7]. The DNA footprinting results suggested 42 bps are covered by HlyU in *V. vulnificus*. However, one dimeric unit of HlyU may not be sufficient to completely cover the whole 42 bps since ~20 bps are covered by IscR whose molecular size is similar to HlyU and operates as a dimer [17]. To minimize the DNA sequence for the binding of *Vv*HlyU dimer, I first divided the DNA sequence into two fragments, fragment Ⅰ and Ⅱ. The DNA fragment designing was implemented for co-crystallization.

To identify the binding to DNA, the size exclusion chromatography (SEC) was conducted using fragment Ⅰ and fragment II. The protein mixture with fragment I showed two peaks in a wide range on a size exclusion chromatography. The peak fractions corresponding to the higher molecular weight contained both *Vv*HlyU and the DNA fragment, as judged by SDS-PAGE and UV/Vis spectrophotometry results (Fig. 3). The peak fraction showed a peak at a wavelength of 260 nm, which is a typical absorption pattern for dsDNA (Fig. 4). These results clearly indicate that *Vv*HlyU binds to 26 bps of dsDNA, shorter than 42 bps. Likewise, DNA fragment Ⅱ was also tested whether it can bind to the *Vv*HlyU protein. When *Vv*HlyU incubated with DNA fragment Ⅱ was applied to a size exclusion chromatographic column, a similar elution profile to that of the protein complex with DNA fragment I was generated. SDS-PAGE and UV/Vis spectrophotometry results demonstrated

the complex formation of *Vv*HlyU and DNA fragment II (Fig. 5). Thus these observation suggests that both the DNA fragments can bind to *Vv*HlyU, and the 42 bps DNA sequence can hold two dimeric units of HlyU.

DNA fragment Ⅰ was chosen for the further structural determination of HlyU and DNA complex. To enhance the chance to obtain the cocrystal, the DNA fragment was trimmed to have a sticky end (table. 1). The DNA-*Vv*HlyU complex was crystallized using hanging drop vapor diffusion method under a precipitation solution containing 0.1 M Tris-HCl (pH 8.5), 8% (*wt*/*vol*) PEG 8K and 2 mM TCEP at 14˚C (Fig. 6). To improve the diffraction quality of the crystal, optimization of the crystallization condition, together with a new construct for the DNA fragment, is now under way.

HlyU



HlyU – DNA fragment I

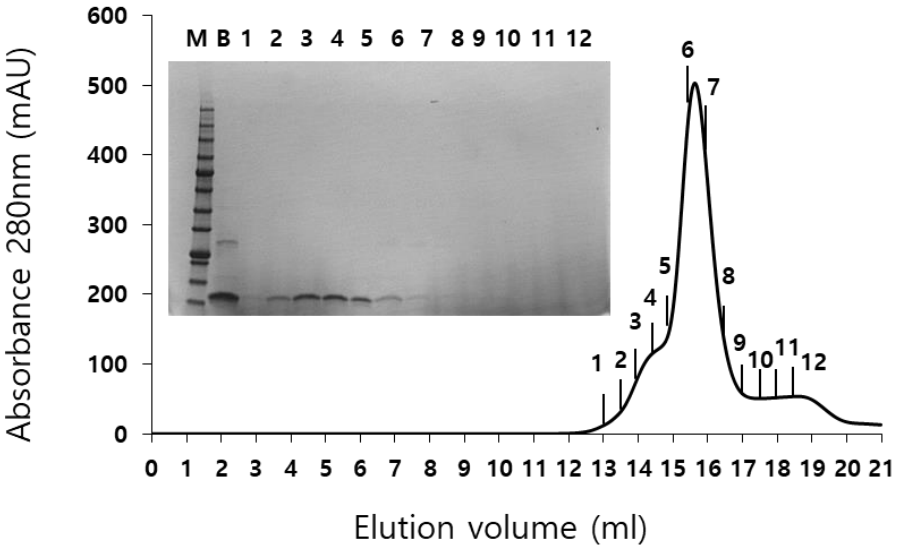


Figure 3. Elution profile from size exclusion chromatography of *Vv*HlyU and *Vv*HlyU-DNA fragment Ⅰ. The fractions from the size exclusion chromatography were used in SDS-PAGE for verification. The fraction numbers are indicated on the peak and image of SDS-PAGE gel. The representative label (M; marker, B; before injection, Number; fraction number) are presented.

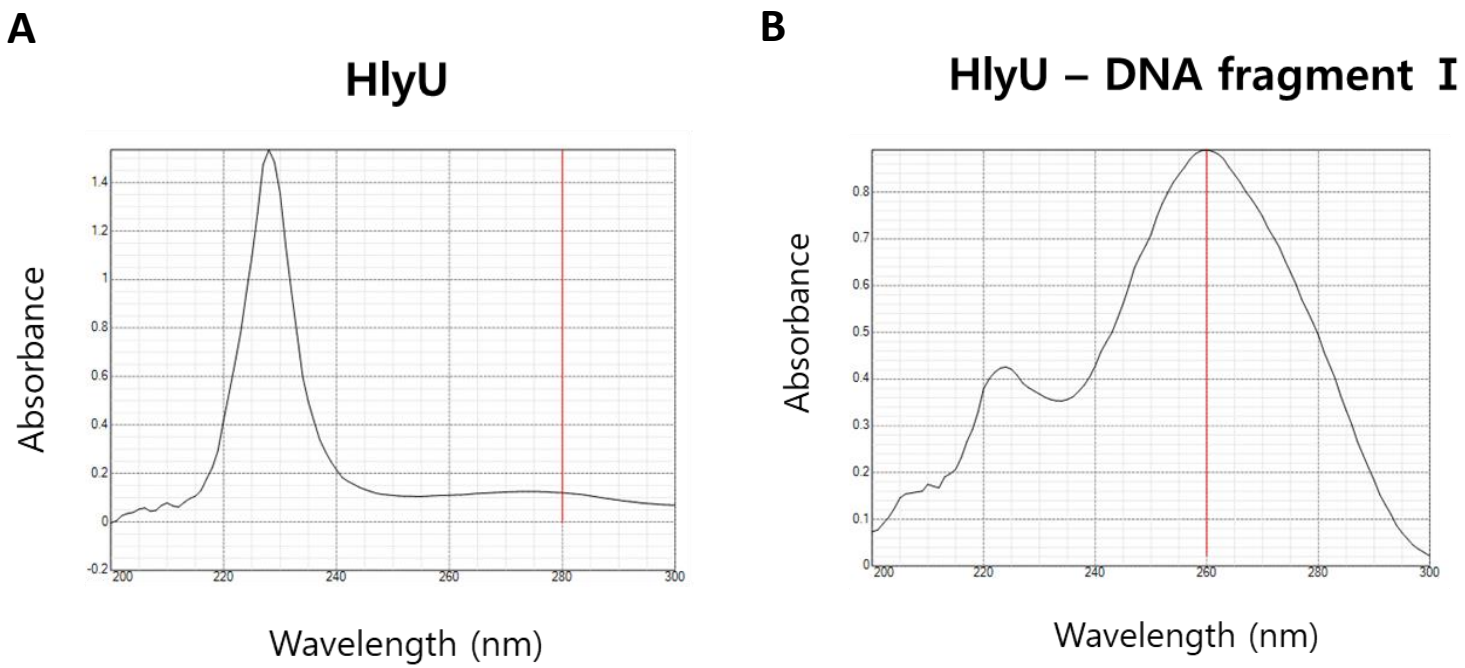
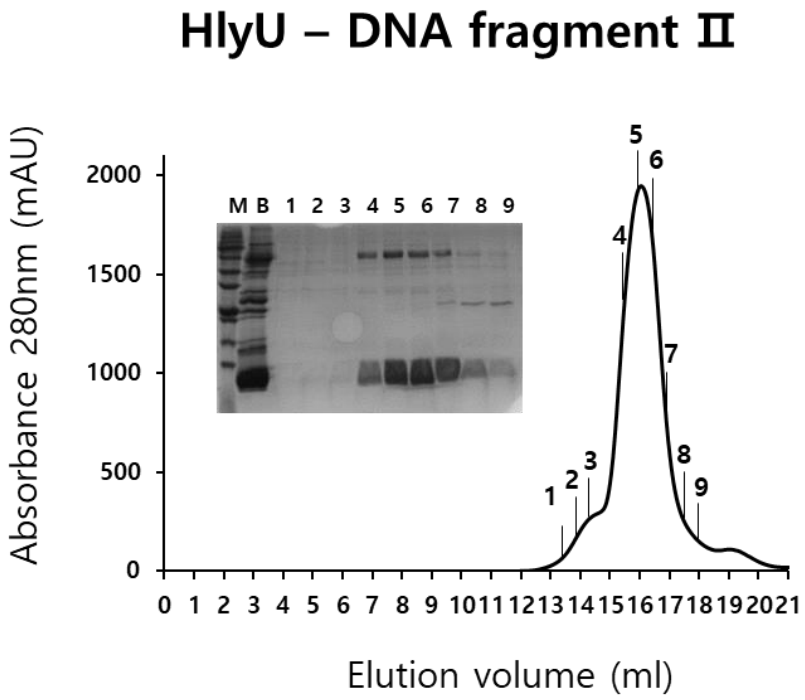


Figure 4. Absorbance profile from spectrophotometer of *Vv*HlyU (A) and with DNA fragment Ⅰ incubated (B).

A


HlyU – DNA fragment Ⅱ
Absorbance 280nm (mAU)
2000
1500
1000
500
0
M B 1 2 3 4 5 6 7 8 9
1
2
3
4
5
6
7
8
9
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21
Elution volume (ml)

B

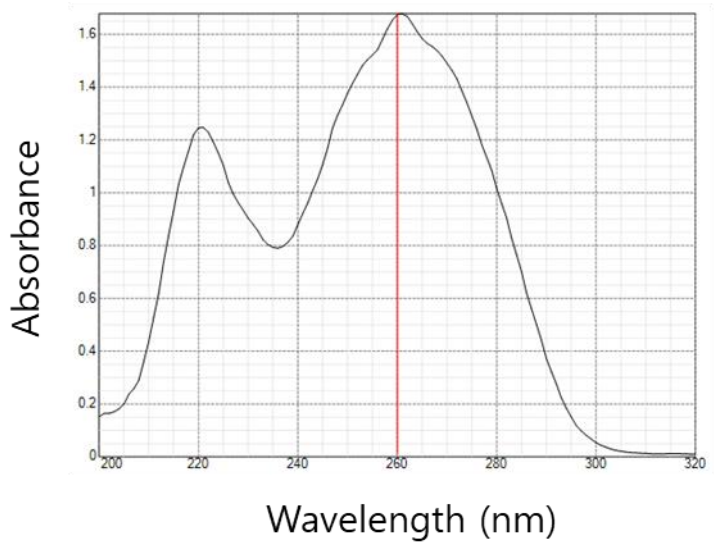

Absorbance
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
200
220
240
260
280
300
320
Wavelength (nm)

Figure 5. Elution profile from size exclusion chromatography (A) and Absorbance profile from spectrometer (B) of *Vv*HlyU-DNA fragment Ⅱ. The fractions from size exclusion chromatography were used in SDS-PAGE for verification. The fraction numbers are indicated on the peak and image of SDS-PAGE gel. The representative label (M; marker, B; before injection, Number; fraction number) are presented.

Table 1. DNA fragments used in experiment.

Fragment I	5′- ATTATTACTTTTTGTTAAATTAGCAT AATAATGAAAAACAATTTAATCGTAT
Fragment II	5′- TTGTTAAATTAGCATTTTCTTCTTTAAATT ACAATTTAATCGTAAAAGAAGAAATTTAAA
Fragment (Final)	5′- TATTAGTTTTTGTTAAATTAGC AATCAAAAACAATTTAATCG

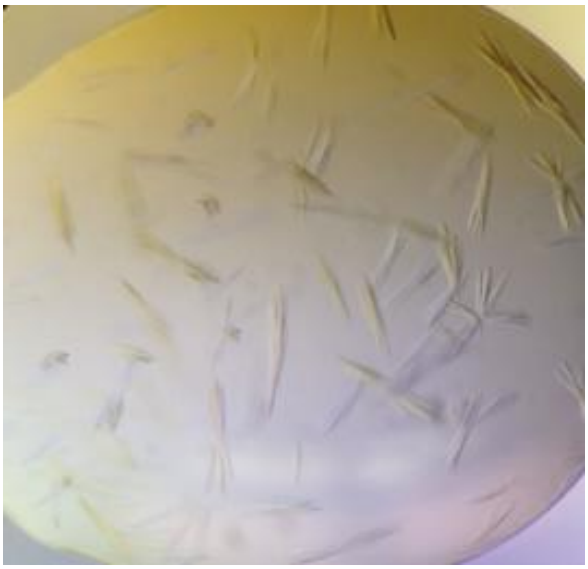


Figure 6. A picture of crystals of *Vv*HlyU protein incubated with DNA fragment (final).

3.3. Crystallization of the *Vv*HlyU protein treated with the compound CM2660

In collaboration with Dr. Sang Ho Choi's Lab in Seoul National University, a synthetic chemical compound N-(4-oxo-4H-thieno[3,4-c]chromen-3-yl)-3-phenylprop-2-ynamide, or shortly CM2660, strongly inhibited the function of *Vv*HlyU when treated to V. vulnificus. Furthermore, the *Vv*HlyU treated with CM2660 abolished the DNA binding ability, as judged by electrophoretic mobility shift assay (EMSA) (Zee-Won Lee and Sang Ho Choi, unpublished data). To obtain the complex protein, the purified HlyU from *V. vulnificus* was incubated with the compound, then the precipitate in the protein mixture was removed by centrifugation. The cubic crystals were initially obtained in a precipitation solution containing 0.1 M HEPES (pH 8.0), 20% (*wt*/*vol*) PEG 4K, and 10% (*vol*/*vol*) 2-propanol. The diffraction-quality crystals were produced by changing the pH and percentage of PEG (Fig. 7).

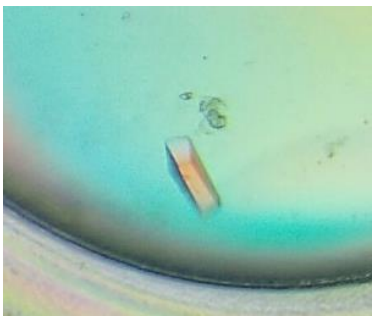


Figure 7. A picture of crystals of *Vv*HlyU protein incubated with CM2660.

3.4. Crystal structure of *Vv*HlyU, incubated with CM2660

The x-ray diffraction dataset was collected at a 2.1 Å resolution at the Pohang Accelerator Laboratory beamline 5C (Fig. 8). The crystals belong to the spacegroup $P4_12_12$ and the unit cell dimensions of a = 35.1 Å , b = 35.1 Å , and c = 180.4 Å . The spacegroup and the cell parameters were dissimilar with those *Vv*HlyU in the absence of the treatment of the compound, previously reported [4]. The crystal structure was solved with molecular replacement model from *V. vulnificus* HlyU (PDB code: 3JTH). The asymmetric unit of the crystal contains monomer, and the final model has an R factor of 0.23% and an R_{free} of 0.29 %.

Like ArsR/SmtB protein family, the crystal structure of *Vv*HlyU has αββα sandwich secondary composition and consists of 5 α-helices and 2 β-strands with loop between two β-strands similar with *Vv* and *Vc*HlyU (Fig. 9). The topology is α1- α2- α3- α4- β1- β2- α5 in order. Unfortunately, the electron density map of the compound was not found in the crystal structure.

In the crystal structure of HlyU from *Vibrio cholerae* [11], an extra density map was also found between two cysteine residues, equivalent to the cysteine residues of *Vv*HlyU. The density was interpreted as a hydroxyl group, resulting in Cys-SOH at Cys36 residue. Moreover, the *Vv*HlyU previously reported [4], contains a water molecule near Cys30, but the density for the water molecule could be interpreted as Cys-SOH as in the case of *Vc*HlyU [11].

Very interestingly, an extra strong density map was observed near the Cys30 and seemed to link to the Cys96 in the crystal structure of *Vv*HlyU in this study (Fig. 10). However, this *Vv*HlyU structure showed a different feature from the previous structures of *Vc*HlyU and *Vv*HlyU. The extra density map of *Vv*HlyU in this study is bigger than the previous ones. Furthermore, the distance between center of density and two cysteine are also different (Fig. 10). From the center of density to Cys30 was 2.2 Å and to another Cys96 was 3.7 Å in *Vv*HlyU in this study. Combined with previous structural studies, it is suggested that the modification of cysteine or interaction between two cysteine occurred.

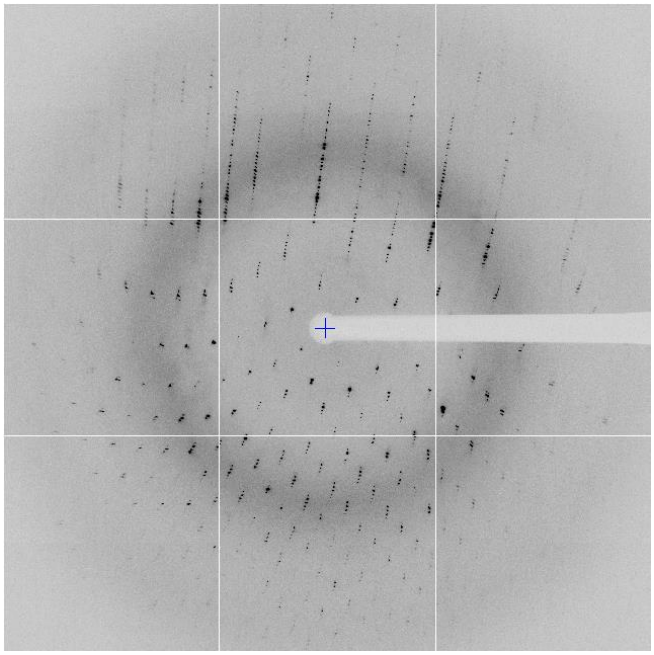


Figure 8. Representative image of X-ray diffraction pattern of *Vv*HlyU protein incubated with CM2660.

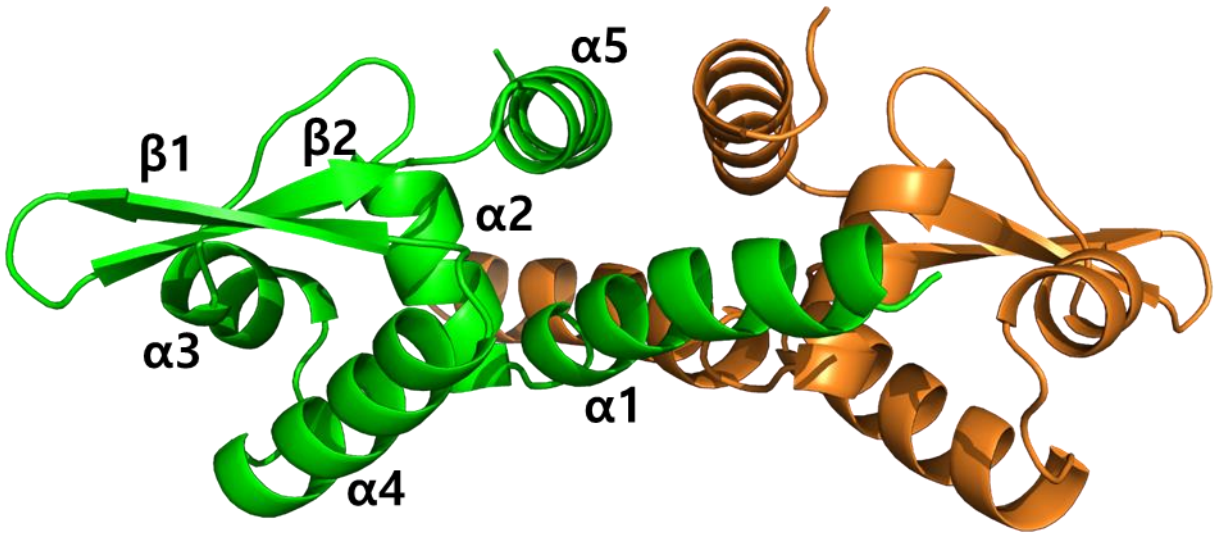


Figure 9. Overall structure of *Vv*HlyU protein incubated with CM2660. Each of the protomers are colored in green and orange, respectively, in ribbon diagram. The elements of secondary structure are labelled.

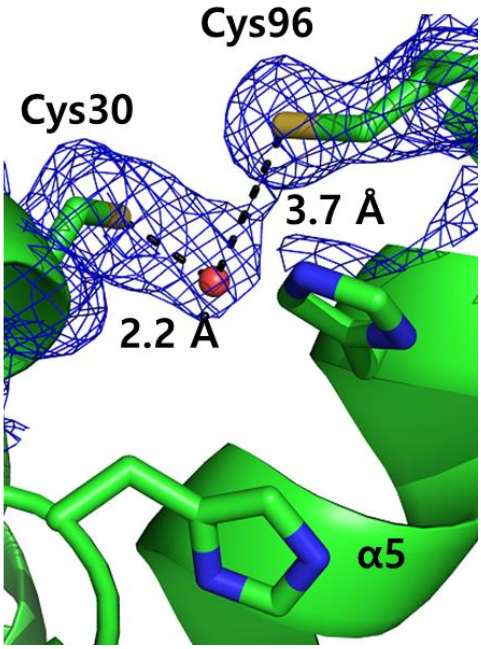


Figure 10. Extra density map between two cysteine. Electron density maps around two cysteine of monomer. The distances between core (red ball) of extra density map and Cys30/Cys96 were displayed with broken black line, respectively. The *F*o-*F*c extra electron density map (blue mesh) around cysteine was contoured at 1.0σ. Residues are displayed in the stick representations and labelled.

Table 2. Statistics for X-ray data collection and refinement

	Native
Data collection	
Beamline	PAL 5C
Wavelength (Å)	0.97960
Space group	$P4_12_12$
Cell dimensions	
a, *b*, *c* (Å)	35.1, 35.1, 180.4
α, β, γ (°)	90, 90, 90
Resolution (Å)	50-2.1 (2.14-2.10)
R_{merge}	0.208 (0.114)
$I/\sigma I$	24.12 (5.64)
Completeness (%)	97.2 (92.2)
Redundancy	19.7 (9.5)
Refinement	
Resolution (Å)	32.74 – 2.11
No. of reflections	6758
R_{work}/R_{free}	0.23/0.29
No. of total atoms	811
Wilson B-factor (Å)	22.60
RMSD	
Bond lengths (Å)	0.007
Bond angles (°)	0.924
Ramachandran plot	
Favored (%)	95.74
Allowed (%)	4.26
Outliers (%)	1.18

*Values in parentheses are for the highest-resolution shell.

** $R_{merge} = \Sigma_{hkl}\Sigma_i|I_i(hkl) - [I(hkl)]|/\Sigma_{hkl}\Sigma_i I_i(hkl)$, where $I_i(hkl)$ is the intensity of the *i*th observation of reflection *hkl* and [I(*hkl*)] is the average intensity of the *i* observations.

*** R_{free} calculated for a random set of 10% of reflections not used in the refinement.

3.5. Structural change of *Vv*HlyU by CM2660

To see the effect of CM2660, this *Vv*HlyU structure (referred to as *Vv*HlyU-CM2660 in this study) was compared with the previous *Vv*HlyU structure whose construct was identical but without any chemical compound [4]. Overall structure of both *Vv*HlyU-CM2660 and *Vv*HlyU are very similar, but the orientation of the subunits in the *Vv*HlyU dimer is changed. Especially, the distance between the α4 helices in *Vv*HlyU-CM2660 were shortened by ~3 Å compared to *Vv*HlyU without the compound. Thus this conformation of *Vv*HlyU-CM2660 is called 'shrink conformation'. Since the α4 helices of the HlyU dimer were previously identified as a DNA recognition motif, the structural change would affect the DNA binding ability or affinity of the HlyU dimer. Furthermore, the C-terminal α5 helices containing Cys96 become closer toward Cys30 in both subunits of the *Vv*HlyU-CM2660 structure by ~ 4 Å in the shrink conformation (Fig. 11). Since the α5 helices is in the dimerization interface, the movement of the helices would result in the inter-subunit movement of *Vv*HlyU.

This residue Also the neighbor residues around two cysteine, His 33 and His 92, were aligned in different way at one of the protomers in the non-incubated structure (Fig. 12). In the details, two histidine were almost identically positioned when superposed onto the incubated structure in the monomer but in the other monomer, two histidine were far away from each other and not matched with the incubated. Combined with the conformation change in dimer and the shortened distance

between two cysteine, Cys30 and Cys96, this study suggested that the shrink form of dimer which regarded as DNA unbound form could be resulted from the two cysteine.

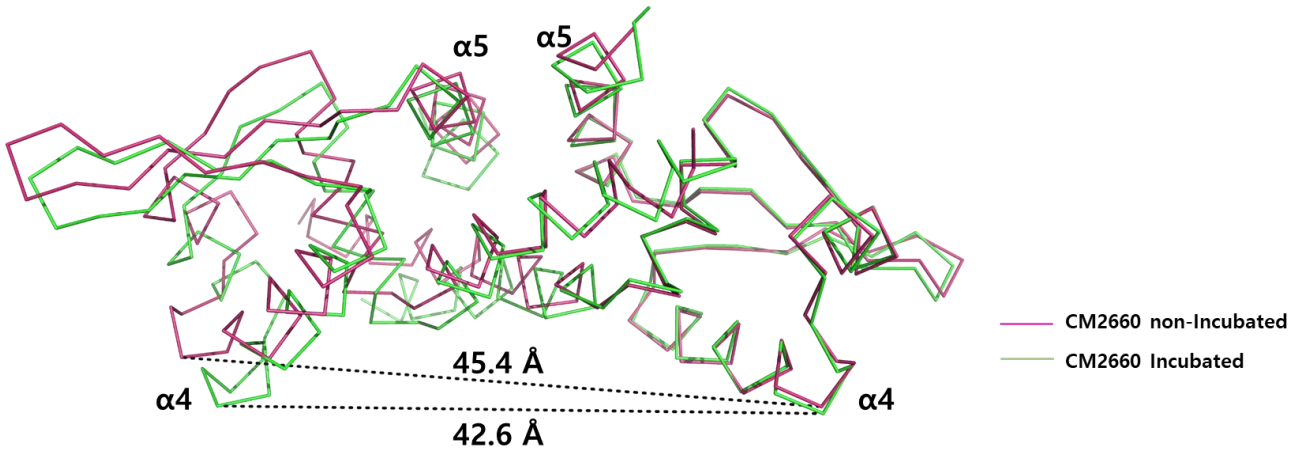


Figure 11. Structural superposition between *Vv*HlyU and *Vv*HlyU protein incubated with CM2660.

Dimer of CM2660 treated with *Vv*HlyU (magenta) and non-treated *Vv*HlyU(green) are aligned structurally in ribbon diagram. The distance between α4 helices of each of dimer are labelled and presented with dashed lines, respectively. Noticeable secondary structure elements are labelled above the corresponding elements.

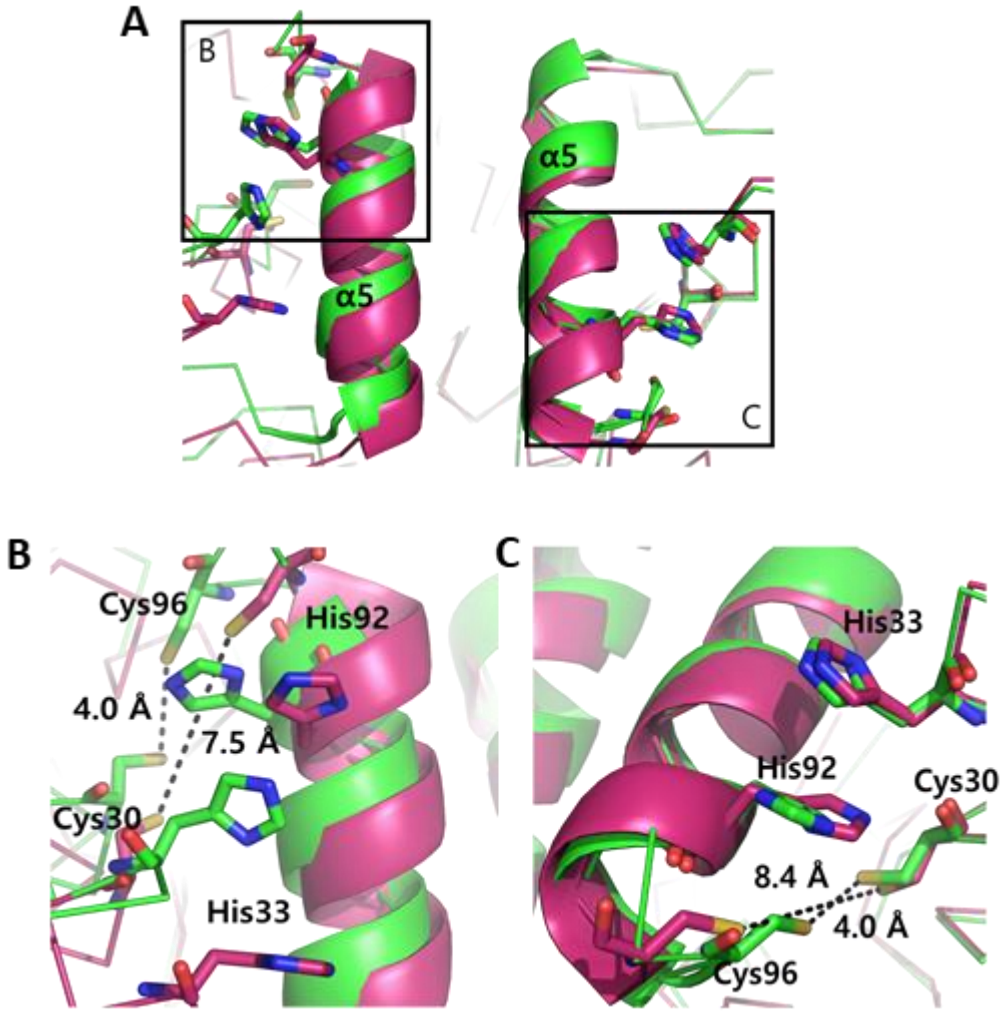


Figure 12. Top view and closed-up view around two cysteine residues in monomer from the superposed *Vv*HlyU and *Vv*HlyU-CM2660.

(A)Top view of dimeric structure from the superposed structures (Fig.11) in ribbon diagram with stick representation. Noticeable secondary structure was labelled. (B, C) Closed-up view of the black box shown in (A) was indicated with the distances and the noticeable residues in stick representation, respectively.

Ⅳ. Discussion

The HlyU has been known as a transcriptional activator in *V. vulnificus*. The RtxA toxin is an important virulence factor for *V. vulnificus* infection and is induced by HlyU by antagonizing the repressor H-NS [7]. This study revealed that the minimum DNA sequence for the HlyU dimer is 22 bps, which was previously known as 42 bps in the promoter of *rtxA* [7, 12], and thus the promoter could hold two copies of the HlyU dimer. Based on the minimum binding sequence, I tried to determine the DNA-complex structure to reveal the molecular mechanism how HlyU recognize DNA and activate the transcription. Despite obtaining the complex crystal of *Vv*HlyU and a DNA fragment, further optimization of crystallization condition is still required to solve the crystal structure.

Any inhibitor study targeting HlyU in *Vibrio* species has never been reported. As a collaborating work, a synthetic compound CM2660 were selected as an antipathogenic agent candidate to reduce the toxin production without inhibiting the growth of the bacteria. Antipathogenic agents are distinguished from antibiotics because they do not select the resistant bacteria and thus is not prone to make the resistance to the agent. Since the treatment of the compound to the purified *Vv*HlyU abolished the DNA binding ability, the direct interaction between the compound and *Vv*HlyU was expected (Sang Ho Choi and Zee-Won Lee unpublished data). In this study, the protein sample, which was treated with the compound, was

successfully crystallized and the crystal structure was determined at a high resolution. The overall structure of *Vv*HlyU treated with the compound was similar to the compound-free *Vv*HlyU structure (PDB code: 3JTH) previously reported [4]. Remarkably, noticeable structural changes were found in the inter-subunit orientation. This study shed a light on the distance between two α4 helices in the dimer. The DNA recognition motif, the α4 helix, in the compound-free HlyU dimer seemed to ideally adjusted to the major grooves of dsDNA in the recognition sequence by an docking study [4]. However, structural superposition revealed that the conformational change by the treatment of the compound resulted in the inward motion of the α4 helix, resulting in change of the DNA binding ability. Combined with the EMSA results, the structural change by the treatment of the compound abolished the DNA binding ability, which may be a crucial step for regulation of the pathogenicity of the bacteria.

Then how the structure of the compound-incubated *Vv*HlyU showed the shortened distance between recognition helices? Further structural analysis suggested that the shrink structure of the compound-incubated *Vv*HlyU protein compared to the compound-free *Vv*HlyU structure is associated with two cysteine residues that become closer to each other. The extra density map near the Cys30 was observed, and it seemed to be linked to Cys96 through one or two covalent bonds, where the compound is responsible because this linkage is expected to be induced by the action of the compound. Cys30 and Cys96 are in the α2 helix and α4 helix, respectively, and the movement of the cysteine residues accompany the motion of

the α-helices α2 and α4. The helix α2 is connected to the wHTH region, and the helix α4 is in a dimeric interface that can affect the inter-subunit orientation. Therefore, the abolishment of DNA binding ability of HlyU by the compound might result from the direct linkage between the two cysteine residues and the following the change in the inter-subunit orientation. Consistently, the structural modification at the wing region and recognition site was previously predicted with MD simulation [12].

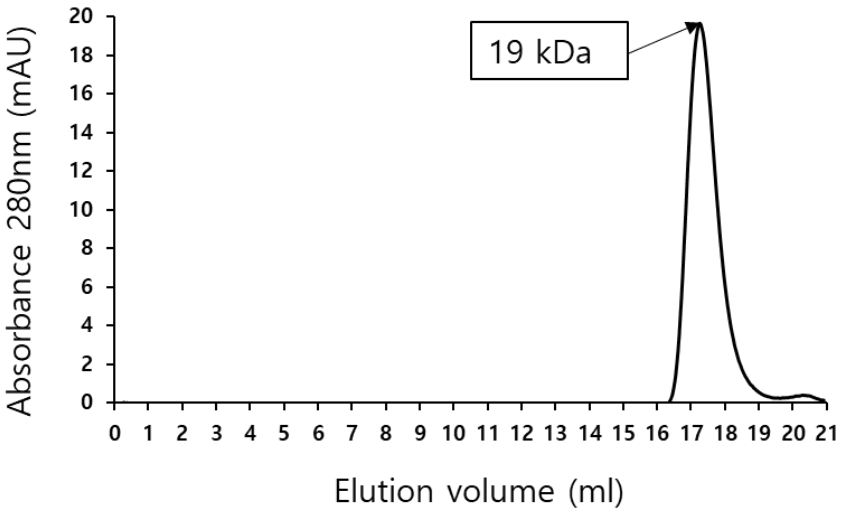
HlyU has been implicated to be activated under the anaerobic condition as a mechanism how *V. vulnificus* senses the host intestine environment where is mostly anaerobic. In the crystal structure of *Vc*HlyU, the equivalent extra density map was also observed when the crystals were grown without any reducing agent. They claimed that sulfur atom would be oxidized since they replaced with CSO (cysteine sulfenic acid) and it fitted in well [11]. It implicates that the cysteine residue could be oxidized very sensitively in response to the oxidative stress. Similarly, CM2660 would function as an oxidant in *Vv*HlyU by promoting oxidation of the two cysteine residues, resulting in 'shrink structure' of *Vv*HlyU. However, the mass analysis is essentially required for the detailed mechanism.

Another possible mechanism of how the compound functions on *Vv*HlyU is to destroy the dimerization of *Vv*HlyU. To test whether CM2660 disrupts the dimerization of *Vv*HlyU, the size exclusion chromatography was conducted (Fig. 13). The treatment of the compound to the *Vv*HlyU protein did not make the

difference in the molecular size of the protein. As a result, I could exclude a possibility that the compound disrupts dimerization of the *Vv*HlyU protein.

In conclusion, the crystal structure *Vv*HlyU with the protein sample treated with the compound. The structure suggests that the compound induces the linkage of the two cysteine residues, resulting in the shift to the shrink conformation of the HlyU dimer that is not optimized for binding to DNA. Based on these observations, the findings in this study could contribute to the development of new drug against *V. vulnificus* infections. It would be a good strategy to do in silico drug discovery with the structure of this study since a drug could fix *Vv*HlyU to this inactive conformation. Since CM2660 can only attenuate the toxicity of the bacteria, this compound is a good candidate for an antipathogenic agent, which is the next-generation antibiotics. Thus if the safety of the compound is confirmed, this compound or its derivatives could be used to prevent the foodborne diseases.

HlyU



HlyU – CM2660

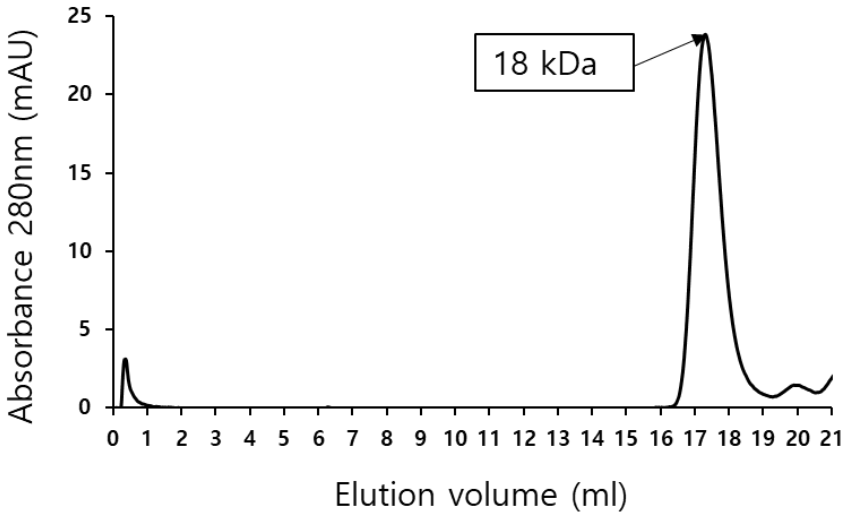


Fig 13. Elution profile of proteins on analytic size exclusion chromatographic column. The calculated molecule sizes of the proteins in solution are labeled on the peak, based on the standard curve (Fig. 1B).

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Ⅵ. 국문 초록

비브리오 패혈증균은 식품 매개의 병원성균으로, 주로 해양성 환경에서 서식한다. 비브리오 패혈증균에 오염된 식품을 섭취하거나 혹은 환부에 직접적으로 접촉되어 감염되면, 심한 장 질환이나 치사율이 50%에 달하는 치명적인 패혈증이 발병한다. 비브리오 패혈증균의 가장 중요한 독성 인자인 RtxA toxin 은 세포에 막세공을 형성함으로써 숙주세포를 사멸시킨다. 이 toxin 의 발현을 조절하는 전사인자인 HlyU 는 호모다이머로 존재하며 ArsR/SmtB 단백질 그룹에 속한다. 현재 비브리오 콜레라균과 비브리오 패혈증균 유래의 HlyU 의 결정 구조가 규명되어있다. 하지만, 현재까지 HlyU 가 어떻게 활성화 되어서 *rtxA* 를 전사 시키는지에 대한 분자적인 작용메커니즘은 밝혀지지 않았다.

비브리오 패혈증균에서 HlyU 의 활성화 시스템을 알기 위해서 HlyU 가 결합하는 DNA 서열의 최소 단위를 밝혀냈으며, 이를 통해 적어도 2 개의 다이머 유닛이 프로모터에 결합하는 것을

확인하였다. 더 나아가 X 선 결정학을 이용하여 DNA 와 단백질의 복합체에 대한 구조 규명을 시도하였다. 복합체의 결정화에 성공하였고, 계속 결정의 조건을 최적화하는 중이다.

최근 다른 연구집단에서 CM2660 이라 명명한 합성화합물을 박테리아에 처리하였을 때 HlyU 의 기능이 저해되는 것을 관찰하였다. 화합물의 저해 메커니즘을 밝혀내기 위하여 화합물을 처리한 HlyU 의 결정화 구조를 규명하였다. 그 결과, HlyU 가 두 개의 유닛으로 DNA 에 결합할 때, 결합에 관여하는 알파 헬릭스 간의 거리가 좁혀지는 구조적 변화가 관찰되었다. 다이머에서 관찰된 구조변화는 단백질과 DNA 의 결합능이 감소되는 것과 연관되어 있는 것으로 보이고, 이로 인해 HlyU 의 기능이 불활성화 된 것으로 분석된다. 비록 화합물과 HlyU 의 결합을 구조에서 확인하지 못했으나, 화합물이 HlyU 다이머 형성을 저해하지 못한다는 것을 알 수 있었다. 화합물을 처리하지 않은 기존의 구조와 비교했을 때, 각 서브유닛에 있는 30 번과 96 번 시스테인 잔기 간의 거리가 감소하였으며, 시스테인 잔기 주변에 추가적인 전자 밀도 맵이 형성된 것도 확인하였다. 종합하여 볼 때,

화합물이 시스테인 잔기에 영향을 미쳐 결과적으로 HlyU 다이머의 구조적인 변화를 야기시킬 것으로 예상된다. 화합물 처리 후 시스테인 잔기의 변화를 분석하기 위해 질량 분석이 필요하다. 본 연구를 통해서 치명적인 식품 매개성 비브리오 패혈증의 발병과 직접적으로 관련 있는 HlyU 의 분자적 수준의 작동메커니즘의 이해에 기여할 수 있었다. 더 나아가 합성화합물의 안전성에 관한 추가연구가 뒷받침된다면 식중독균의 항독력제로 식품산업에 이용가치가 충분히 있을 것이라 기대된다.

주요어: 패혈증 비브리오균, 전사인자, HlyU, 저해제 후보, 결정 구조
학번: 2015-23133

A THESIS FOR THE DEGREE OF MASTER OF SCIENCE

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비브리오 패혈증균 독성 발현 전사 인자 HlyU의 구조적 분석

August, 2017

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석사학위논문

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지도교수 하 남 출

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서울대학교 대학원

농생명공학부

박 노 라

박노라의 석사학위논문을 인준함

2017년 8월

위원장 유상렬 **(인)**

부위원장 하남출 **(인)**

위원 강동현 **(인)**

Abstract

Vibrio vulnificus is a foodborne pathogenic bacterium that is present in marine environments. The infection results from uptake of contaminated food or direct contacts on the injured skin, causing severe gastroenteritis or fatal septicemia in a mortality rate of more than 50% within a couple of days. The most important virulence factor of *V. vulnificus* is a RtxA toxin that can kill the host cells by forming pores on the membrane. Induction of RtxA toxin is mediated by a transcriptional regulator HlyU, which belongs to ArsR/SmtB protein family that operates as a homodimer. To date, the crystal structures of HlyU from *Vibrio cholera* and *V. vulnificus* have been determined. However, it remains elucidated how HlyU is activated and is participated in transcription of *rtxA* at a molecular level. To reveal the mechanism of action of HlyU in *V. vulnificus*, I first determined a minimal DNA sequence for binding of HlyU, indicating that at least two units of the HlyU dimer is bound to the promoter region of rtxA1. Subsequently, the structural determination of the complex of DNA-*Vv*HlyU was tried by x-ray crystallography. The crystals of complex of HlyU and the DNA fragment were obtained, and the optimization of the crystallization conditions is now in progress to determine the crystal structure.

Recently, a research group in this department discovered a synthetic chemical compound called CM2660 inhibits the function of HlyU when the compound was treated to the bacteria. In order to identify mechanism of how CM2660 inhibits the function of HlyU, I determined crystal structure of HlyU from *V. vulnificus* with a protein sample treated with CM2660. A significant change of the inter-subunit orientation by the compound was found with the reduced interval of the two DNA binding α-helices in the HlyU dimer. This conformational change in the dimer is expected to be associated with a lower binding ability to DNA, leading to inactivation of the HlyU function. Although the compound itself was not observed in the crystal structure, the distance between two cysteine, Cys30 and Cys96 in each subunit, was shortened when compared to the compound-free structure previously reported. In addition, I further identified that CM2660 did not disrupt the dimeric form of HlyU and the presence of extra electron density map between Cys30 and Cys96. Taken together, it is expected that CM2660 may promote the interaction or modification at Cys30 and consequently result in conformational change in the dimer. In order to analyze modification of the cysteine residues, mass spectrometric analysis is under way. This study contributes to the understanding of the HlyU molecular mechanism, which is directly related to the pathogenicity of the fatal foodborne bacterium *V. vulnificus*.

***Keywords*: *Vibrio vulnificus*, transcriptional activator, HlyU, putative inhibitor, crystal structure**

***Student Number:* 2015-23133**

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Ⅰ. Introduction

Vibrio vulnificus is an aerobic gram-negative bacterium, which is highly pathogenic to human. It commonly lives in marine environments and in the human body [1, 2]. *V. vulnificus* is highly cytotoxic to eukaryotic cells, and this cytotoxicity is generally regarded as the hallmark of the pathogenicity of this microorganism [3]. Exposure to contaminated water or ingestion of contaminated food or direct administration of the bacteria to injured skin can cause severe gastroenteritis or fatal septicemia with a rapid progress, resulting in a mortality rate of more than 50% within a couple of days [4].

There are two major cytotoxins identified in this organism: cytolysin/hemolysin (encoded by *vvhA*) and RTX (repeats in toxin; encoded by *rtxA1*). The RTX toxin forms pores on cell membranes only after the contact of the bacterium with the host cell. This action of the toxin is required for *V. vulnificus* virulence in mice by promoting bacterial colonization at the infection site and subsequent invasion into the bloodstream [5]. Moreover, the decreased cytotoxic activity and the virulence due to a mutation in the *rtxA* gene were observed in other research, implicating its critical role in the cytotoxic activity and virulence of *V. vulnificus* [4]. Recently, Liu et al. reported that activation of the *rtxA* gene expression is controlled by HlyU at the transcription initiation level [6]. Furthermore, the transcription of the RTX

operon was decreased in a *hlyU* mutant through transcriptome analysis using DNA chip [3]. The activity of HlyU has been confirmed by in vivo-induced antigen technology and proved that HlyU is essential for *V. vulnificus* virulence in mice [5]. HlyU binds to the promoter region which is preoccupied by H-NS, a global repressor in many bacteria, leading to depression of the repression function of H-NS [7]. Accordingly, HlyU has been implicated as an important transcriptional activator of the virulence factors by de-repressing the function of H-NS factors and common in *Vibrio* species [8-10].

The transcriptional activator HlyU belongs to ArsR/SmtB protein family, which is small regulatory proteins containing a helix-turn-helix motif [8]. Most ArsR/SmtB protein family members has a metal binding site, which is important in turning on the transcriptional activity by capturing the metal ion in it. Dissimilar to ArsR/SmtB protein family, HlyU is likely not to have the corresponding metal binding since the residues lining the metal binding site are replaced in HlyU. Therefore, it's a plausible explanation that HlyU might not be involved in the metal binding for its function.

To date, the crystal structures of HlyU from *Vibrio vulnificus* and *Vibrio cholerae* have been determined [11]. HlyU from *V. vulnificus* (*Vv*HlyU) shares 78% sequence identity with HlyU from *V. cholerae* (*Vc*HlyU), exhibiting similar secondary structure elements. In the details of structure of *Vc*HlyU, the Cys38 of *Vc*HlyU and its equivalent residue Cys30 of *Vv*HlyU were noted. The *Vc*HlyU structures

represented two modification states at Cys38. In the presence of a reducing agent tris(2-carboxyethyl) phosphine (TCEP), the extra electron density map near Cys38 wasn't observed in *Vc*HlyU structure. Remarkably, an electron density map close to the sulfur atom of the cysteine residue was found and considered as modification of cysteine to CSO, in an oxidized form of *Vc*HlyU in the absence of reducing agent [11]. Therefore, it seemed that Cys38 of *Vc*HlyU is sensitive to oxidative stress, and its oxidation is limited in oxygen-limiting environments, such as intestine in human. The sensitive oxidation of Cys38 suggests a potential of HlyU as thiol-based redox switches. Thus, this finding would be applicable to the *Vv*HlyU.

According to the crystal structures of *Vv*HlyU and *Vc*HlyU, important residues playing role in making a dimeric interface or secondary structure elements for the binding were observed. Especially, among those residues, the importance of cysteine residues was emphasized. The deduction in distance between two cysteine which is presented on dimeric interface in both of *Vc*HlyU and *Vv*HlyU (Cys38 and Cys104 for the *V. cholerae* and Cys30 and Cys96 for the *V. vulnificus*) caused structural modification on the wing and the recognition α helix contacting DNA and resulted in DNA-unbound form. It is in consistency with result of MD simulation from *V. vulnificus [12]*.

A previous research has identified that HlyU binds directly to a region upstream of the operon where rtxA1 is located [5]. During the preparation of the experiment, I had doubt about the size of binding sequence which is too long for the HlyU

dimeric unit to cover. I speculated that at least two HlyU dimeric units might be bound to the promoter region sequentially to cover the long DNA sequence [4, 13].

In this study, I found the minimum binding unit of a HlyU dimer and also determined the structure of *Vv*HlyU incubated with a chemical compound, N- (4- oxo- 4H- thieno[3,4- c]chromen- 3- yl)- 3- phenylprop- 2- ynamide, or shortly CM2660, as a putative inhibitor. This structure further was analyzed with mass focusing on cysteine and its neighboring residues regarded as major components causing conformational changes.

Ⅱ. Materials and Methods

2.1. Plasmid construction

The template DNA of the HlyU from *V. vulnificus* was kindly provided by Dr. Jeong-Sun Kim at Cheonnam University [4]. The recombinant plasmid (pProEx-HTc-*Vv*HlyU) was transformed into *Escherichia coli* strain C43(DE3) [14] using heat-shock method and incubated overnight at 37°C for protein production.

2.2. DNA fragment

To define the minimal binding unit, I divided into two fragments of DNA from the binding sequence discovered by Liu *et al*. The two fragments were then oligo synthesized (table. 1). These each forward and reverse DNA fragments were annealed by decreasing temperature slowly from 45°C results in dsDNA, suitable form for DNA-protein complex. The modified fragment Ⅰ was used for crystallization.

2.3. Purification of *Vv*HlyU

2.3.1. Overexpression

The recombinant HlyU was inoculated by picking a single colony from *E.coli* strain

into 25ml of LB medium with 50μg/ml ampicillin (Duchefa, Netherlands) and incubated at 37°C overnight. The *E. coli* strain was cultured in 2.0 L of LB medium including appropriate antibiotics until an OD_{600} of 0.8 and protein production was induced with 0.5 mM IPTG at 30℃. Cells were harvested 6 h after induction, and the cell pellet was resuspended with 50 ml of lysis buffer containing 20 mM Tris-HCl (pH 7.5), 500 mM NaCl, and 2 mM 2-mercaptoethanol. After homogenization by Sonicator, the cell lysate was acquired by centrifugation at 13,000 rpm for 30 min.

2.3.2. Affinity chromatography

The 6X His-tagged HlyU protein of the cell lysate was bound to Ni^{2+}-NTA resin by rolling for 1 h at 4°C. The Ni^{2+}-NTA resin bound with recombinant HlyU washed out with buffer containing 500 mM NaCl, 20 mM Tris-HCl (pH 7.5), 40 mM Imidazole (pH 7.5), and 2 mM β-mercaptoethanol to remove the other proteins. Elution was carried out by using lysis buffer containing 250 mM Imidazole (pH 7.5). The size and purity of eluted protein was identified by SDS-PAGE.

2.3.3. Size exclusion chromatography (SEC)

The eluted proteins were further purified by size exclusion chromatography (HiLoad Superdex 200 26/600; GE Healthcare). The column was pre-equilibrated with buffer containing 300 mM NaCl, 20 mM Tris-HCl (pH 7.5), and 2 mM β-

mercaptoethanol. The fractions corresponding to HlyU were concentrated up to 9 mg/ml using a centrifugal filter concentration device (Millipore, Temecula, CA; 10 kDa cutoff). The purity of protein was confirmed by SDS-PAGE. After the concentration, the proteins were incubated with chemical compound, putative inhibitor, by 1:10 molecular ratio for 30 min on ice. They were further centrifuged at 13,000 rpm for 7 min to remove the pellet made during the incubation if it was necessary. The final incubated sample was stored frozen at -80℃.

Also each of the binding DNA fragment Ⅰ/Ⅱ were incubated with proteins by 1: 1.2 to 1.5 mole ratio for 30 min at 4°C. Before the incubation, 0.1mM final concentration of divalent ions such as magnesium chloride were used to protein for an hour at 4°C to assist making stabilized dsDNA complex form. The mixture applied to column again for separating bound form from unbound form. The fractions identified as bound form were centrifuged and stored under -80℃.

2.4. Crystallization

Crystallization of HlyU incubated with CM2660 and DNA-HlyU complex were performed using the vapor-diffusion hanging drop method at 14℃ under a mother liquor containing 0.1 M Tris-HCl (pH 8.5), 8% (*wt*/*vol*) PEG 8K for the DNA-HlyU complex and 0.1M HEPES (pH 8), 20% (*wt*/*vol*) PEG 4K, and 10% (*vol*/*vol*) 2-propanol for CM2660-incubated HlyU. The compound incubated crystals were

flash-frozen using 20% (*vol*/*vol*) sorbitol as a cryoprotectant in a nitrogen stream at -173℃ prior to collecting the X-ray diffraction dataset with the Pohang Accelerator Laboratory beamline 5C and were processed with the HKL2000 package [15].

2.5. Structural determination and refinement

The crystals of the compound-incubated *Vv*HlyU belonged to the spacegroup $P4_12_12$, with unit cell dimensions of a = 35.1 Å , b = 35.1 Å , and c = 180.4 Å (Table 2). The structure was determined using the MOLREP program in the CCP4 package by the molecular replacement method and a search model taken from the HlyU from *V. vulnificus* CMCP6 (PDB code: 3JTH)[4]. The final structure of HlyU incubated with compound was refined at a 2.1 Å resolution with an R factor of 0.23% and an R_{free} of 0.29% using the PHENIX program [16]. Further details on the structure determination and refinement are given in Table 2.

2.6. Analytic size exclusion chromatography

To find out whether the compound is responsible for disrupting dimerization of HlyU or not, size exclusion chromatography using a HiLoad Superdex GL 200 26/600 column (GE Healthcare). The column was pre-calibrated with following size standard proteins: ferritin (440 kDa), aldolase (158 kDa), conalbumin (75 kDa), ovalbumin (44 kDa), carbonic anhydrase (29 kDa), and RNase A (13.7 kDa). The

samples treated with the compound or not were then applied to the column and eluted fractions at the peak were electrophoresed to confirm the molecular size of HlyU.

2.7. Identification of DNA-protein complex

The proteins incubated with dsDNA fragments by 1:1.5 molar ratio for 30 min at 4℃ were eluted from size exclusion chromatography as described above. The fraction considered as complex were measured spectrophotometrically using spectrophotometer in a range from 200 nm to 300 nm.

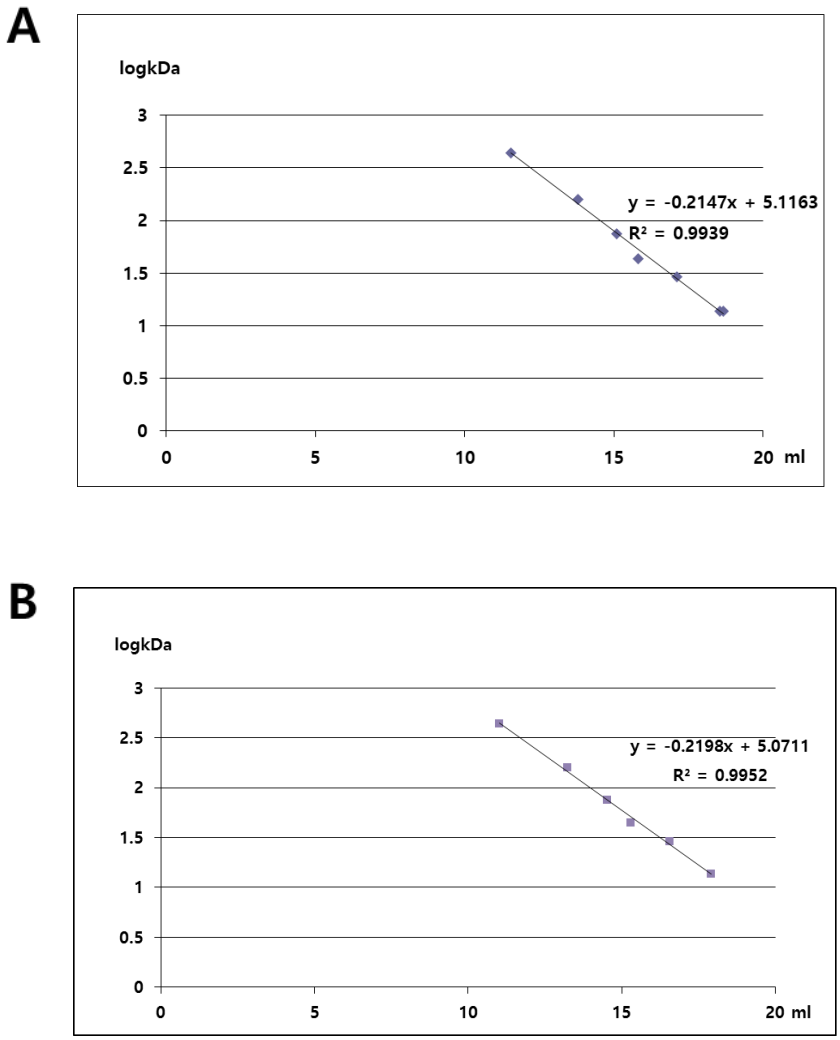


Figure 1. Standard curve of analytic size exclusion chromatography

(A) Used in experiment of *Vv*HlyU-DNA, (B) Used in experiment of *Vv*HlyU-CM2660. Analytic size exclusion chromatography was calibrated based on the following standard proteins represented in triangle: ferritin (440 kDa), aldolase (158 kDa), conalbumin (75 kDa), ovalbumin (44 kDa), carbonic anhydrase (29 kDa), and RNase A (13.7 kDa).

Ⅲ. Results

3.1. Overexpression and purification of *Vv*HlyU

The crystal structure of *V. vulnificus* HlyU was previously determined [4]. However, the structure of the DNA-protein complex and the inhibitor-protein complex has not been characterized yet. Fortunately, the expression vector for *Vv*HlyU was kindly provided by Dr. Jeong-Sun Kim at Cheonnam University, and the expression level was sufficient for the following studies. The recombinant protein was successfully produced in the *E. coli* expression system. The HlyU, 6X His-tagged protein at N-terminus, was purified by affinity chromatography. It was further purified by size exclusion chromatography. The SDS-PAGE was carried out at each step to verify the purities of the samples (Fig. 2).

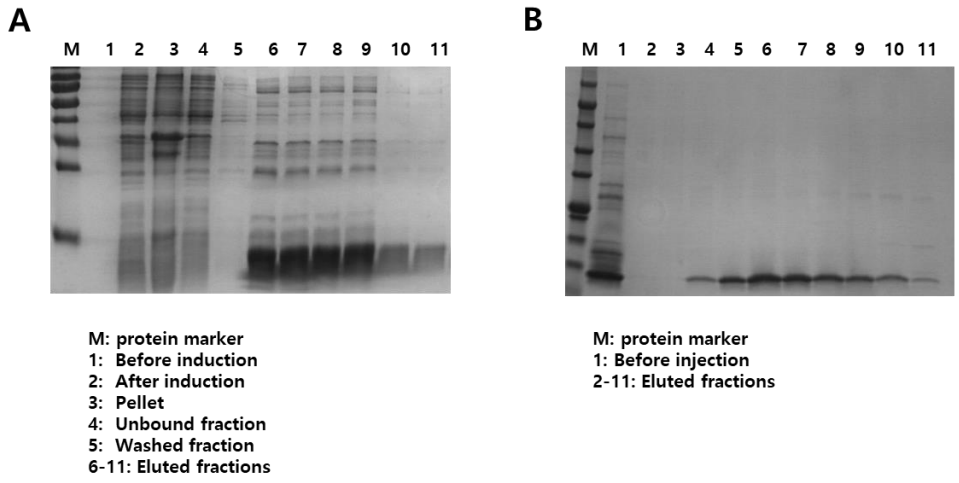


Figure 2. Elution profile from Ni-NTA affinity chromatography (A) and Size exclusion chromatography (B).

3.2. Minimal unit of DNA for binding to *Vv*HlyU

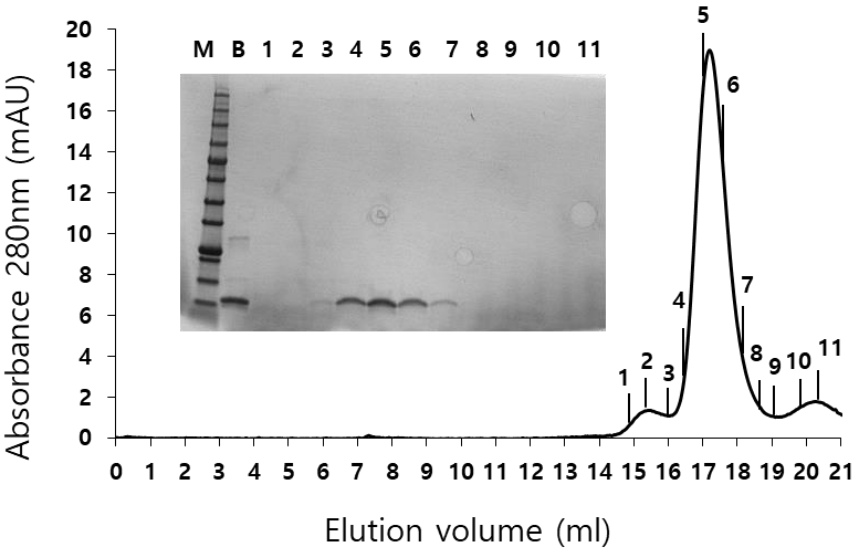
Binding site of *Vv*HlyU at promoter region was previously revealed by another group [7]. The DNA footprinting results suggested 42 bps are covered by HlyU in *V. vulnificus*. However, one dimeric unit of HlyU may not be sufficient to completely cover the whole 42 bps since ~20 bps are covered by IscR whose molecular size is similar to HlyU and operates as a dimer [17]. To minimize the DNA sequence for the binding of *Vv*HlyU dimer, I first divided the DNA sequence into two fragments, fragment Ⅰ and Ⅱ. The DNA fragment designing was implemented for co-crystallization.

To identify the binding to DNA, the size exclusion chromatography (SEC) was conducted using fragment Ⅰ and fragment II. The protein mixture with fragment I showed two peaks in a wide range on a size exclusion chromatography. The peak fractions corresponding to the higher molecular weight contained both *Vv*HlyU and the DNA fragment, as judged by SDS-PAGE and UV/Vis spectrophotometry results (Fig. 3). The peak fraction showed a peak at a wavelength of 260 nm, which is a typical absorption pattern for dsDNA (Fig. 4). These results clearly indicate that *Vv*HlyU binds to 26 bps of dsDNA, shorter than 42 bps. Likewise, DNA fragment Ⅱ was also tested whether it can bind to the *Vv*HlyU protein. When *Vv*HlyU incubated with DNA fragment Ⅱ was applied to a size exclusion chromatographic column, a similar elution profile to that of the protein complex with DNA fragment I was generated. SDS-PAGE and UV/Vis spectrophotometry results demonstrated

the complex formation of *Vv*HlyU and DNA fragment II (Fig. 5). Thus these observation suggests that both the DNA fragments can bind to *Vv*HlyU, and the 42 bps DNA sequence can hold two dimeric units of HlyU.

DNA fragment Ⅰ was chosen for the further structural determination of HlyU and DNA complex. To enhance the chance to obtain the cocrystal, the DNA fragment was trimmed to have a sticky end (table. 1). The DNA-*Vv*HlyU complex was crystallized using hanging drop vapor diffusion method under a precipitation solution containing 0.1 M Tris-HCl (pH 8.5), 8% (*wt*/*vol*) PEG 8K and 2 mM TCEP at 14˚C (Fig. 6). To improve the diffraction quality of the crystal, optimization of the crystallization condition, together with a new construct for the DNA fragment, is now under way.

HlyU



HlyU – DNA fragment I

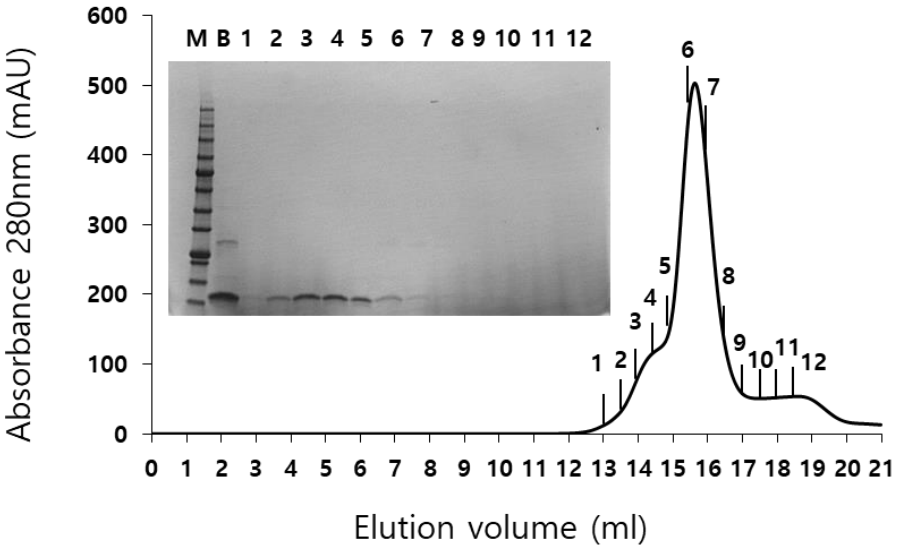


Figure 3. Elution profile from size exclusion chromatography of *Vv*HlyU and *Vv*HlyU-DNA fragment Ⅰ. The fractions from the size exclusion chromatography were used in SDS-PAGE for verification. The fraction numbers are indicated on the peak and image of SDS-PAGE gel. The representative label (M; marker, B; before injection, Number; fraction number) are presented.

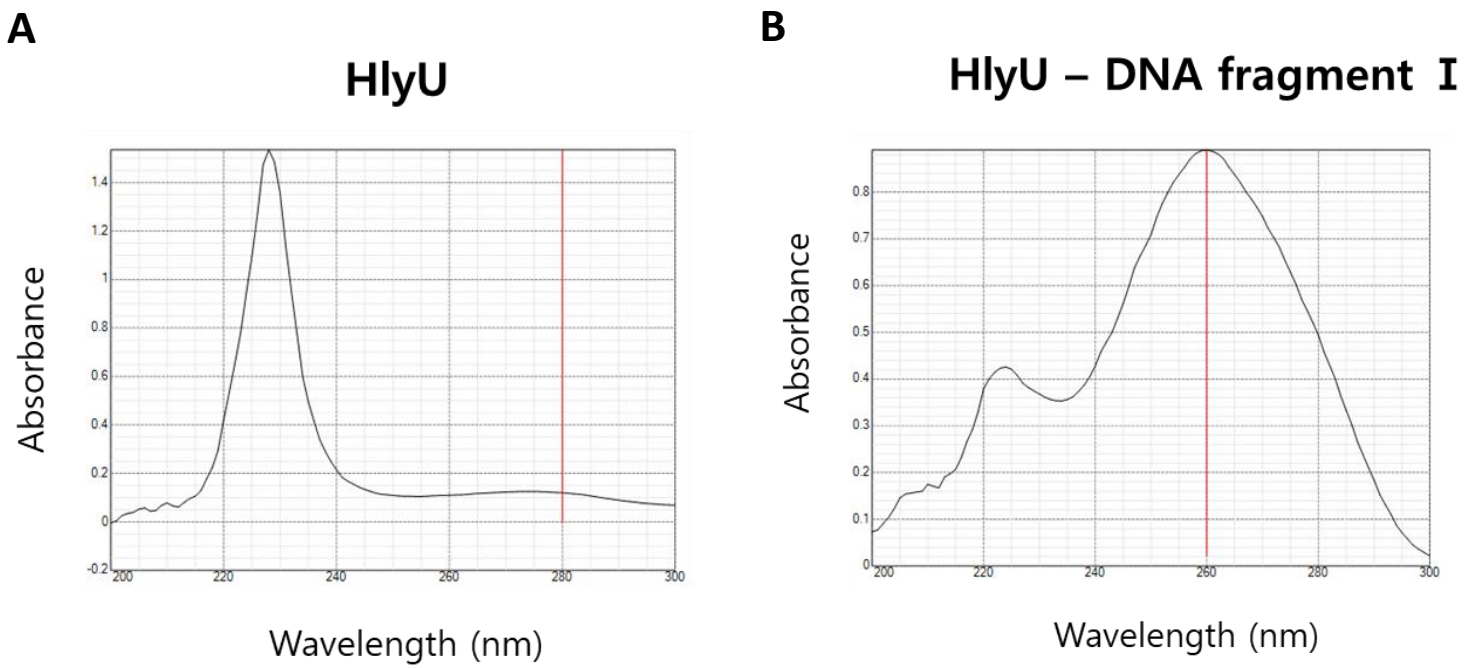
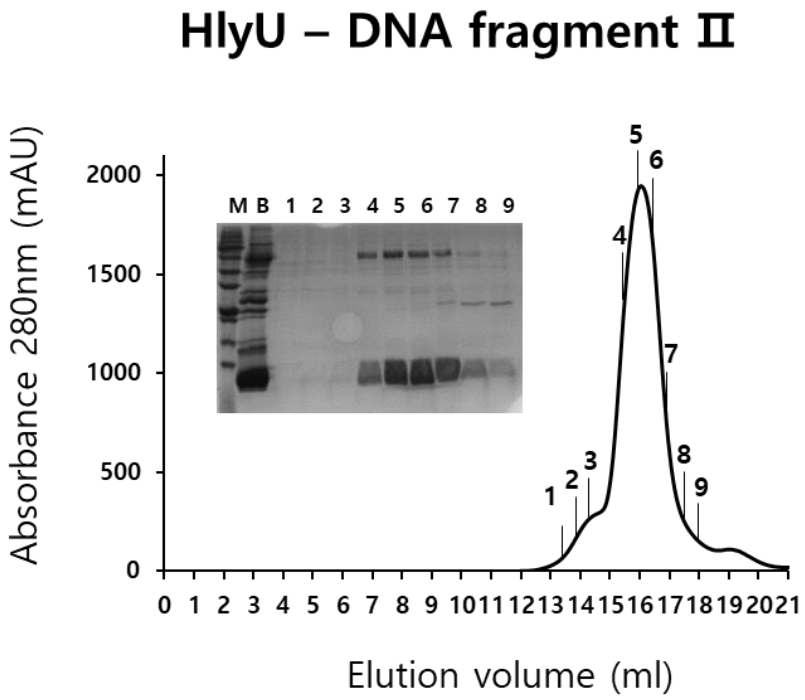


Figure 4. Absorbance profile from spectrophotometer of *Vv*HlyU (A) and with DNA fragment Ⅰ incubated (B).

A


HlyU – DNA fragment Ⅱ
Absorbance 280nm (mAU)
2000
1500
1000
500
0
M B 1 2 3 4 5 6 7 8 9
1
2
3
4
5
6
7
8
9
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21
Elution volume (ml)

B

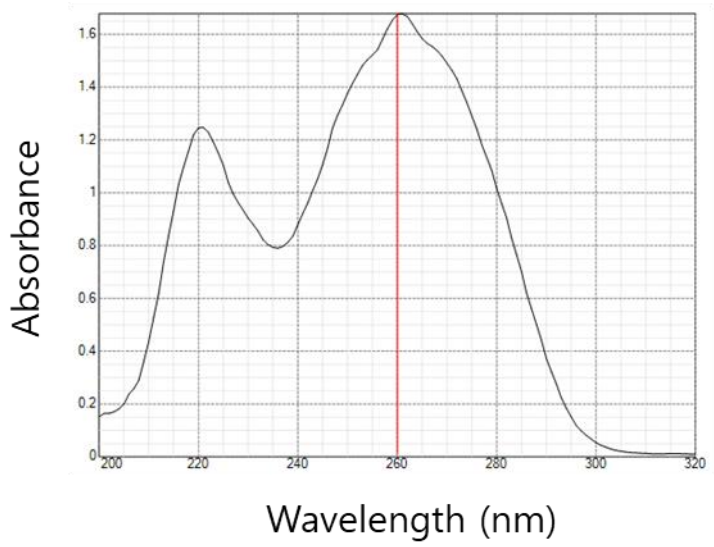

Absorbance
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
200
220
240
260
280
300
320
Wavelength (nm)

Figure 5. Elution profile from size exclusion chromatography (A) and Absorbance profile from spectrometer (B) of *Vv*HlyU-DNA fragment Ⅱ. The fractions from size exclusion chromatography were used in SDS-PAGE for verification. The fraction numbers are indicated on the peak and image of SDS-PAGE gel. The representative label (M; marker, B; before injection, Number; fraction number) are presented.

Table 1. DNA fragments used in experiment.

Fragment I	5′- ATTATTACTTTTTGTTAAATTAGCAT AATAATGAAAAACAATTTAATCGTAT
Fragment II	5′- TTGTTAAATTAGCATTTTCTTCTTTAAATT ACAATTTAATCGTAAAAGAAGAAATTTAAA
Fragment (Final)	5′- TATTAGTTTTTGTTAAATTAGC AATCAAAAACAATTTAATCG

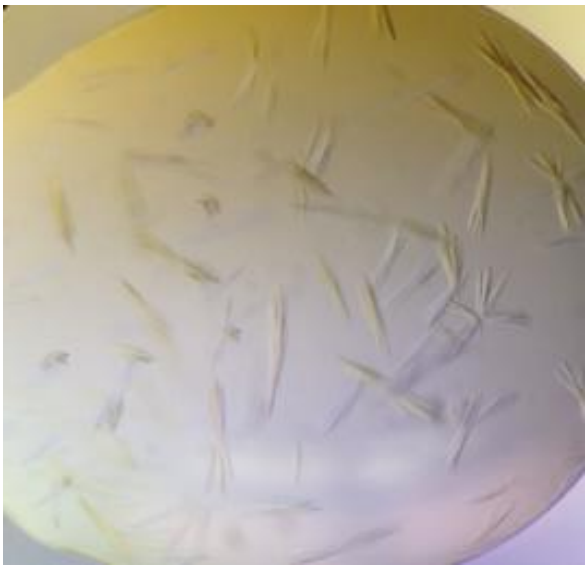


Figure 6. A picture of crystals of *Vv*HlyU protein incubated with DNA fragment (final).

3.3. Crystallization of the *Vv*HlyU protein treated with the compound CM2660

In collaboration with Dr. Sang Ho Choi's Lab in Seoul National University, a synthetic chemical compound N-(4-oxo-4H-thieno[3,4-c]chromen-3-yl)-3-phenylprop-2-ynamide, or shortly CM2660, strongly inhibited the function of *Vv*HlyU when treated to V. vulnificus. Furthermore, the *Vv*HlyU treated with CM2660 abolished the DNA binding ability, as judged by electrophoretic mobility shift assay (EMSA) (Zee-Won Lee and Sang Ho Choi, unpublished data). To obtain the complex protein, the purified HlyU from *V. vulnificus* was incubated with the compound, then the precipitate in the protein mixture was removed by centrifugation. The cubic crystals were initially obtained in a precipitation solution containing 0.1 M HEPES (pH 8.0), 20% (*wt*/*vol*) PEG 4K, and 10% (*vol*/*vol*) 2-propanol. The diffraction-quality crystals were produced by changing the pH and percentage of PEG (Fig. 7).

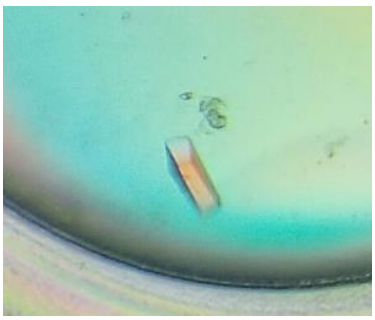


Figure 7. A picture of crystals of *Vv*HlyU protein incubated with CM2660.

3.4. Crystal structure of *Vv*HlyU, incubated with CM2660

The x-ray diffraction dataset was collected at a 2.1 Å resolution at the Pohang Accelerator Laboratory beamline 5C (Fig. 8). The crystals belong to the spacegroup $P4_12_12$ and the unit cell dimensions of a = 35.1 Å , b = 35.1 Å , and c = 180.4 Å . The spacegroup and the cell parameters were dissimilar with those *Vv*HlyU in the absence of the treatment of the compound, previously reported [4]. The crystal structure was solved with molecular replacement model from *V. vulnificus* HlyU (PDB code: 3JTH). The asymmetric unit of the crystal contains monomer, and the final model has an R factor of 0.23% and an R_{free} of 0.29 %.

Like ArsR/SmtB protein family, the crystal structure of *Vv*HlyU has αββα sandwich secondary composition and consists of 5 α-helices and 2 β-strands with loop between two β-strands similar with *Vv* and *Vc*HlyU (Fig. 9). The topology is α1- α2- α3- α4- β1- β2- α5 in order. Unfortunately, the electron density map of the compound was not found in the crystal structure.

In the crystal structure of HlyU from *Vibrio cholerae* [11], an extra density map was also found between two cysteine residues, equivalent to the cysteine residues of *Vv*HlyU. The density was interpreted as a hydroxyl group, resulting in Cys-SOH at Cys36 residue. Moreover, the *Vv*HlyU previously reported [4], contains a water molecule near Cys30, but the density for the water molecule could be interpreted as Cys-SOH as in the case of *Vc*HlyU [11].

Very interestingly, an extra strong density map was observed near the Cys30 and seemed to link to the Cys96 in the crystal structure of *Vv*HlyU in this study (Fig. 10). However, this *Vv*HlyU structure showed a different feature from the previous structures of *Vc*HlyU and *Vv*HlyU. The extra density map of *Vv*HlyU in this study is bigger than the previous ones. Furthermore, the distance between center of density and two cysteine are also different (Fig. 10). From the center of density to Cys30 was 2.2 Å and to another Cys96 was 3.7 Å in *Vv*HlyU in this study. Combined with previous structural studies, it is suggested that the modification of cysteine or interaction between two cysteine occurred.

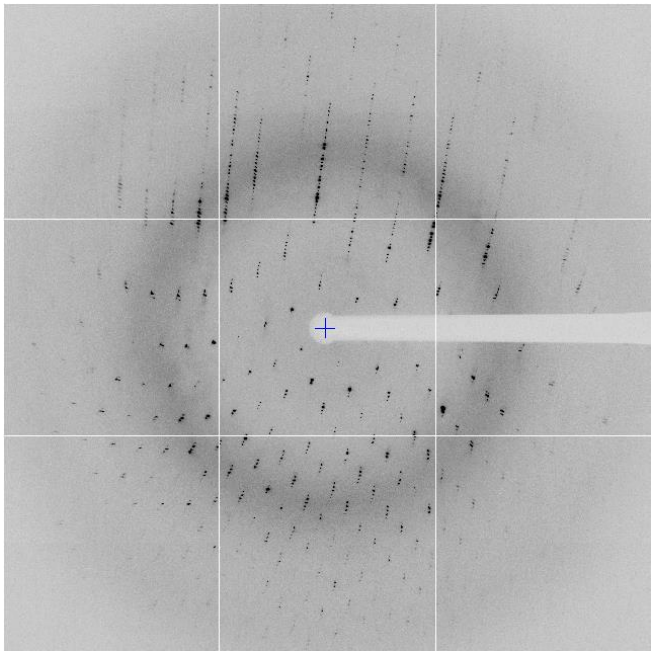


Figure 8. Representative image of X-ray diffraction pattern of *Vv*HlyU protein incubated with CM2660.

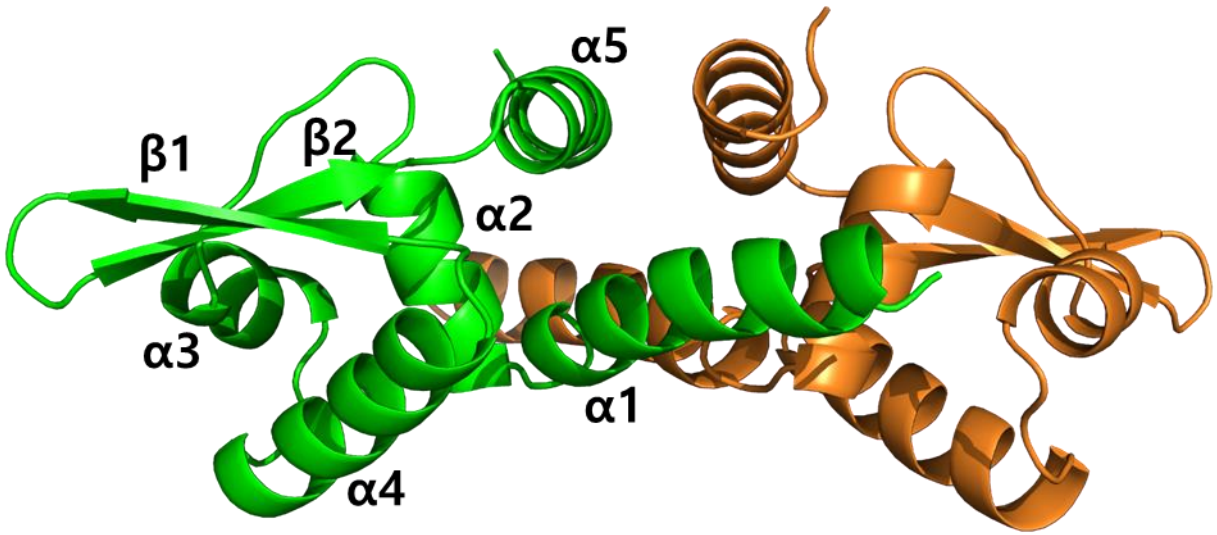


Figure 9. Overall structure of *Vv*HlyU protein incubated with CM2660. Each of the protomers are colored in green and orange, respectively, in ribbon diagram. The elements of secondary structure are labelled.

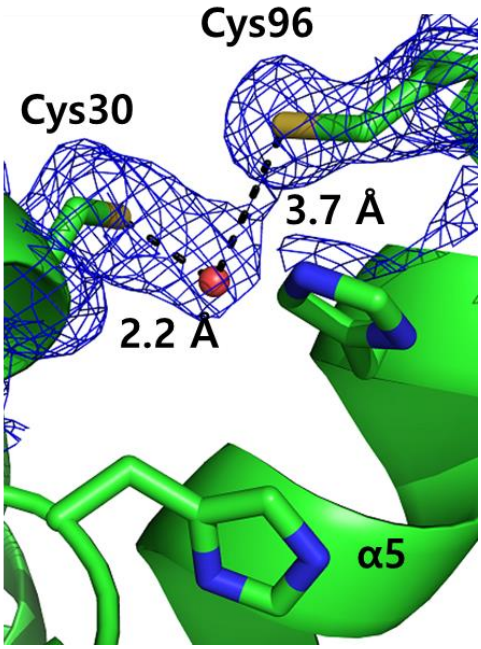


Figure 10. Extra density map between two cysteine. Electron density maps around two cysteine of monomer. The distances between core (red ball) of extra density map and Cys30/Cys96 were displayed with broken black line, respectively. The *F*o-*F*c extra electron density map (blue mesh) around cysteine was contoured at 1.0σ. Residues are displayed in the stick representations and labelled.

Table 2. Statistics for X-ray data collection and refinement

	Native
Data collection	
Beamline	PAL 5C
Wavelength (Å)	0.97960
Space group	$P4_12_12$
Cell dimensions	
a, *b*, *c* (Å)	35.1, 35.1, 180.4
α, β, γ (°)	90, 90, 90
Resolution (Å)	50-2.1 (2.14-2.10)
R_{merge}	0.208 (0.114)
$I/\sigma I$	24.12 (5.64)
Completeness (%)	97.2 (92.2)
Redundancy	19.7 (9.5)
Refinement	
Resolution (Å)	32.74 – 2.11
No. of reflections	6758
R_{work}/R_{free}	0.23/0.29
No. of total atoms	811
Wilson B-factor (Å)	22.60
RMSD	
Bond lengths (Å)	0.007
Bond angles (°)	0.924
Ramachandran plot	
Favored (%)	95.74
Allowed (%)	4.26
Outliers (%)	1.18

*Values in parentheses are for the highest-resolution shell.

** $R_{merge} = \Sigma_{hkl}\Sigma_i|I_i(hkl) - [I(hkl)]|/\Sigma_{hkl}\Sigma_i I_i(hkl)$, where $I_i(hkl)$ is the intensity of the *i*th observation of reflection *hkl* and [I(*hkl*)] is the average intensity of the *i* observations.

*** R_{free} calculated for a random set of 10% of reflections not used in the refinement.

3.5. Structural change of *Vv*HlyU by CM2660

To see the effect of CM2660, this *Vv*HlyU structure (referred to as *Vv*HlyU-CM2660 in this study) was compared with the previous *Vv*HlyU structure whose construct was identical but without any chemical compound [4]. Overall structure of both *Vv*HlyU-CM2660 and *Vv*HlyU are very similar, but the orientation of the subunits in the *Vv*HlyU dimer is changed. Especially, the distance between the α4 helices in *Vv*HlyU-CM2660 were shortened by ~3 Å compared to *Vv*HlyU without the compound. Thus this conformation of *Vv*HlyU-CM2660 is called 'shrink conformation'. Since the α4 helices of the HlyU dimer were previously identified as a DNA recognition motif, the structural change would affect the DNA binding ability or affinity of the HlyU dimer. Furthermore, the C-terminal α5 helices containing Cys96 become closer toward Cys30 in both subunits of the *Vv*HlyU-CM2660 structure by ~ 4 Å in the shrink conformation (Fig. 11). Since the α5 helices is in the dimerization interface, the movement of the helices would result in the inter-subunit movement of *Vv*HlyU.

This residue Also the neighbor residues around two cysteine, His 33 and His 92, were aligned in different way at one of the protomers in the non-incubated structure (Fig. 12). In the details, two histidine were almost identically positioned when superposed onto the incubated structure in the monomer but in the other monomer, two histidine were far away from each other and not matched with the incubated. Combined with the conformation change in dimer and the shortened distance

between two cysteine, Cys30 and Cys96, this study suggested that the shrink form of dimer which regarded as DNA unbound form could be resulted from the two cysteine.

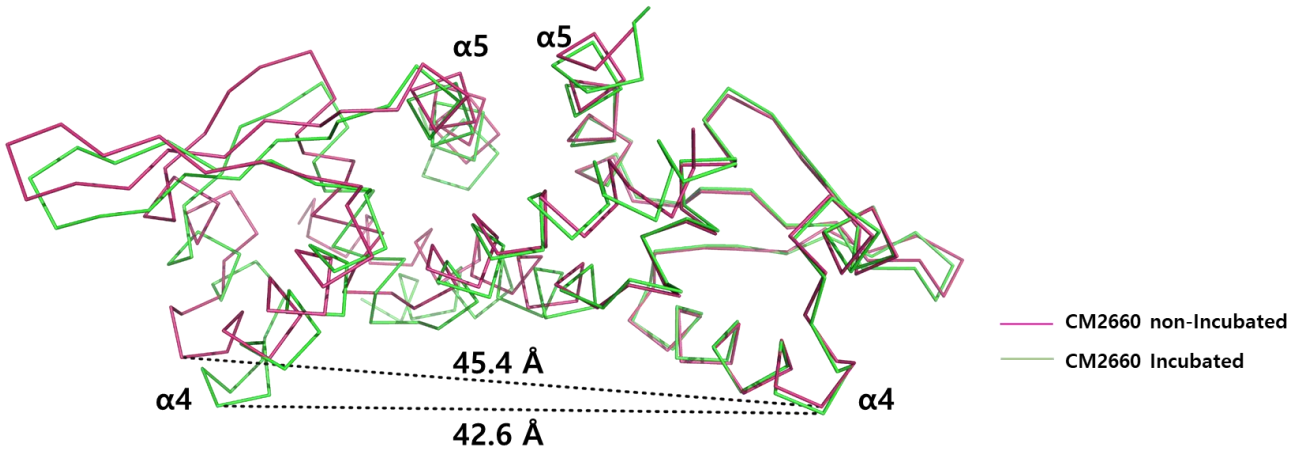


Figure 11. Structural superposition between *Vv*HlyU and *Vv*HlyU protein incubated with CM2660.

Dimer of CM2660 treated with *Vv*HlyU (magenta) and non-treated *Vv*HlyU(green) are aligned structurally in ribbon diagram. The distance between α4 helices of each of dimer are labelled and presented with dashed lines, respectively. Noticeable secondary structure elements are labelled above the corresponding elements.

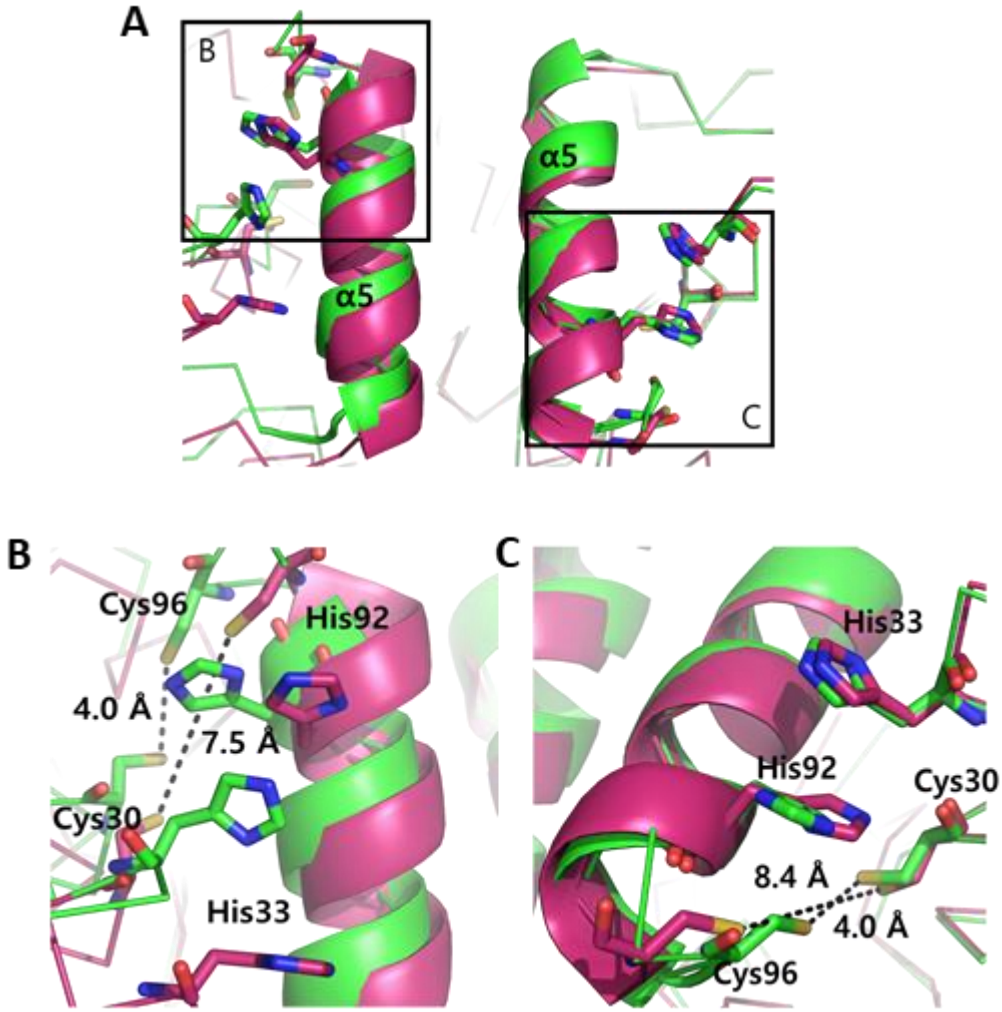


Figure 12. Top view and closed-up view around two cysteine residues in monomer from the superposed *Vv*HlyU and *Vv*HlyU-CM2660.

(A)Top view of dimeric structure from the superposed structures (Fig.11) in ribbon diagram with stick representation. Noticeable secondary structure was labelled. (B, C) Closed-up view of the black box shown in (A) was indicated with the distances and the noticeable residues in stick representation, respectively.

Ⅳ. Discussion

The HlyU has been known as a transcriptional activator in *V. vulnificus*. The RtxA toxin is an important virulence factor for *V. vulnificus* infection and is induced by HlyU by antagonizing the repressor H-NS [7]. This study revealed that the minimum DNA sequence for the HlyU dimer is 22 bps, which was previously known as 42 bps in the promoter of *rtxA* [7, 12], and thus the promoter could hold two copies of the HlyU dimer. Based on the minimum binding sequence, I tried to determine the DNA-complex structure to reveal the molecular mechanism how HlyU recognize DNA and activate the transcription. Despite obtaining the complex crystal of *Vv*HlyU and a DNA fragment, further optimization of crystallization condition is still required to solve the crystal structure.

Any inhibitor study targeting HlyU in *Vibrio* species has never been reported. As a collaborating work, a synthetic compound CM2660 were selected as an antipathogenic agent candidate to reduce the toxin production without inhibiting the growth of the bacteria. Antipathogenic agents are distinguished from antibiotics because they do not select the resistant bacteria and thus is not prone to make the resistance to the agent. Since the treatment of the compound to the purified *Vv*HlyU abolished the DNA binding ability, the direct interaction between the compound and *Vv*HlyU was expected (Sang Ho Choi and Zee-Won Lee unpublished data). In this study, the protein sample, which was treated with the compound, was

successfully crystallized and the crystal structure was determined at a high resolution. The overall structure of *Vv*HlyU treated with the compound was similar to the compound-free *Vv*HlyU structure (PDB code: 3JTH) previously reported [4]. Remarkably, noticeable structural changes were found in the inter-subunit orientation. This study shed a light on the distance between two α4 helices in the dimer. The DNA recognition motif, the α4 helix, in the compound-free HlyU dimer seemed to ideally adjusted to the major grooves of dsDNA in the recognition sequence by an docking study [4]. However, structural superposition revealed that the conformational change by the treatment of the compound resulted in the inward motion of the α4 helix, resulting in change of the DNA binding ability. Combined with the EMSA results, the structural change by the treatment of the compound abolished the DNA binding ability, which may be a crucial step for regulation of the pathogenicity of the bacteria.

Then how the structure of the compound-incubated *Vv*HlyU showed the shortened distance between recognition helices? Further structural analysis suggested that the shrink structure of the compound-incubated *Vv*HlyU protein compared to the compound-free *Vv*HlyU structure is associated with two cysteine residues that become closer to each other. The extra density map near the Cys30 was observed, and it seemed to be linked to Cys96 through one or two covalent bonds, where the compound is responsible because this linkage is expected to be induced by the action of the compound. Cys30 and Cys96 are in the α2 helix and α4 helix, respectively, and the movement of the cysteine residues accompany the motion of

the α-helices α2 and α4. The helix α2 is connected to the wHTH region, and the helix α4 is in a dimeric interface that can affect the inter-subunit orientation. Therefore, the abolishment of DNA binding ability of HlyU by the compound might result from the direct linkage between the two cysteine residues and the following the change in the inter-subunit orientation. Consistently, the structural modification at the wing region and recognition site was previously predicted with MD simulation [12].

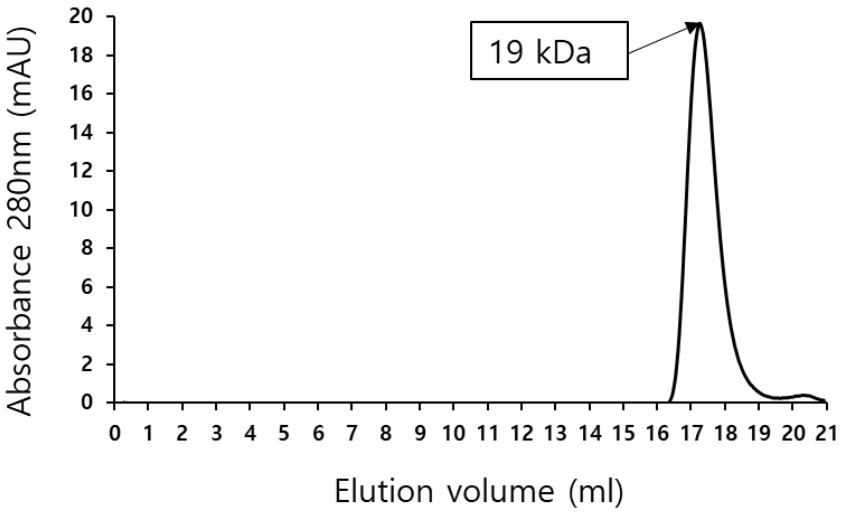
HlyU has been implicated to be activated under the anaerobic condition as a mechanism how *V. vulnificus* senses the host intestine environment where is mostly anaerobic. In the crystal structure of *Vc*HlyU, the equivalent extra density map was also observed when the crystals were grown without any reducing agent. They claimed that sulfur atom would be oxidized since they replaced with CSO (cysteine sulfenic acid) and it fitted in well [11]. It implicates that the cysteine residue could be oxidized very sensitively in response to the oxidative stress. Similarly, CM2660 would function as an oxidant in *Vv*HlyU by promoting oxidation of the two cysteine residues, resulting in 'shrink structure' of *Vv*HlyU. However, the mass analysis is essentially required for the detailed mechanism.

Another possible mechanism of how the compound functions on *Vv*HlyU is to destroy the dimerization of *Vv*HlyU. To test whether CM2660 disrupts the dimerization of *Vv*HlyU, the size exclusion chromatography was conducted (Fig. 13). The treatment of the compound to the *Vv*HlyU protein did not make the

difference in the molecular size of the protein. As a result, I could exclude a possibility that the compound disrupts dimerization of the *Vv*HlyU protein.

In conclusion, the crystal structure *Vv*HlyU with the protein sample treated with the compound. The structure suggests that the compound induces the linkage of the two cysteine residues, resulting in the shift to the shrink conformation of the HlyU dimer that is not optimized for binding to DNA. Based on these observations, the findings in this study could contribute to the development of new drug against *V. vulnificus* infections. It would be a good strategy to do in silico drug discovery with the structure of this study since a drug could fix *Vv*HlyU to this inactive conformation. Since CM2660 can only attenuate the toxicity of the bacteria, this compound is a good candidate for an antipathogenic agent, which is the next-generation antibiotics. Thus if the safety of the compound is confirmed, this compound or its derivatives could be used to prevent the foodborne diseases.

HlyU



HlyU – CM2660

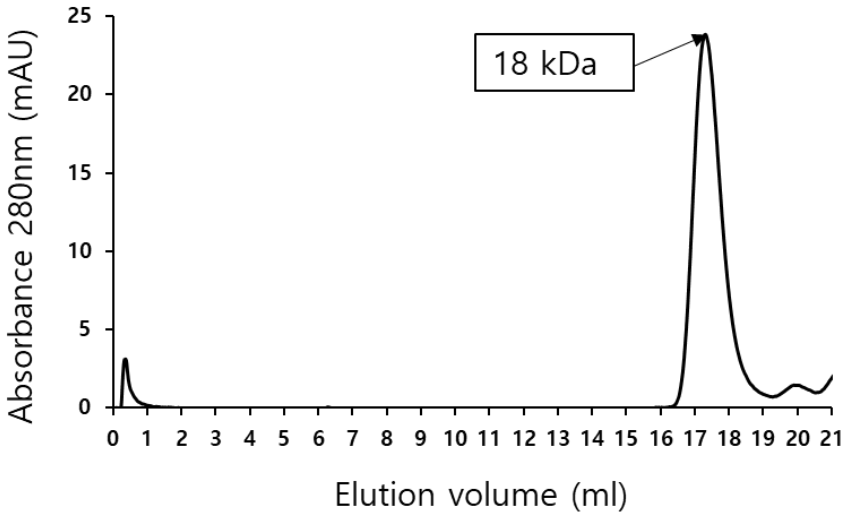


Fig 13. Elution profile of proteins on analytic size exclusion chromatographic column. The calculated molecule sizes of the proteins in solution are labeled on the peak, based on the standard curve (Fig. 1B).

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Ⅵ. 국문 초록

비브리오 패혈증균은 식품 매개의 병원성균으로, 주로 해양성 환경에서 서식한다. 비브리오 패혈증균에 오염된 식품을 섭취하거나 혹은 환부에 직접적으로 접촉되어 감염되면, 심한 장 질환이나 치사율이 50%에 달하는 치명적인 패혈증이 발병한다. 비브리오 패혈증균의 가장 중요한 독성 인자인 RtxA toxin 은 세포에 막세공을 형성함으로써 숙주세포를 사멸시킨다. 이 toxin 의 발현을 조절하는 전사인자인 HlyU 는 호모다이머로 존재하며 ArsR/SmtB 단백질 그룹에 속한다. 현재 비브리오 콜레라균과 비브리오 패혈증균 유래의 HlyU 의 결정 구조가 규명되어있다. 하지만, 현재까지 HlyU 가 어떻게 활성화 되어서 *rtxA* 를 전사 시키는지에 대한 분자적인 작용메커니즘은 밝혀지지 않았다.

비브리오 패혈증균에서 HlyU 의 활성화 시스템을 알기 위해서 HlyU 가 결합하는 DNA 서열의 최소 단위를 밝혀냈으며, 이를 통해 적어도 2 개의 다이머 유닛이 프로모터에 결합하는 것을

확인하였다. 더 나아가 X 선 결정학을 이용하여 DNA 와 단백질의 복합체에 대한 구조 규명을 시도하였다. 복합체의 결정화에 성공하였고, 계속 결정의 조건을 최적화하는 중이다.

최근 다른 연구집단에서 CM2660 이라 명명한 합성화합물을 박테리아에 처리하였을 때 HlyU 의 기능이 저해되는 것을 관찰하였다. 화합물의 저해 메커니즘을 밝혀내기 위하여 화합물을 처리한 HlyU 의 결정화 구조를 규명하였다. 그 결과, HlyU 가 두 개의 유닛으로 DNA 에 결합할 때, 결합에 관여하는 알파 헬릭스 간의 거리가 좁혀지는 구조적 변화가 관찰되었다. 다이머에서 관찰된 구조변화는 단백질과 DNA 의 결합능이 감소되는 것과 연관되어 있는 것으로 보이고, 이로 인해 HlyU 의 기능이 불활성화 된 것으로 분석된다. 비록 화합물과 HlyU 의 결합을 구조에서 확인하지 못했으나, 화합물이 HlyU 다이머 형성을 저해하지 못한다는 것을 알 수 있었다. 화합물을 처리하지 않은 기존의 구조와 비교했을 때, 각 서브유닛에 있는 30 번과 96 번 시스테인 잔기 간의 거리가 감소하였으며, 시스테인 잔기 주변에 추가적인 전자 밀도 맵이 형성된 것도 확인하였다. 종합하여 볼 때,

화합물이 시스테인 잔기에 영향을 미쳐 결과적으로 HlyU 다이머의 구조적인 변화를 야기시킬 것으로 예상된다. 화합물 처리 후 시스테인 잔기의 변화를 분석하기 위해 질량 분석이 필요하다. 본 연구를 통해서 치명적인 식품 매개성 비브리오 패혈증의 발병과 직접적으로 관련 있는 HlyU 의 분자적 수준의 작동메커니즘의 이해에 기여할 수 있었다. 더 나아가 합성화합물의 안전성에 관한 추가연구가 뒷받침된다면 식중독균의 항독력제로 식품산업에 이용가치가 충분히 있을 것이라 기대된다.

주요어: 패혈증 비브리오균, 전사인자, HlyU, 저해제 후보, 결정 구조
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