약학석사 학위논문

Structural and functional study on HEPN-MNT proteins from *Legionella pneumophila*

*Legionella pneumophila*에서 유래한
HEPN-MNT 단백질의 구조 및 기능 연구

2021년 2월

서울대학교 대학원
약학과 물리약학전공

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이 논문을 약학석사 학위논문으로 제출함

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Abstract

Structural and functional study on HEPN-MNT proteins from *Legionella pneumophila*

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Legionella pneumophila belongs to pathogenic bacteria which is classified as gram-negative bacteria. One of the representative species is *Legionella pneumophila*. About 20 percent of bacterial pneumonia in the world is known to be caused by *Legionella* species. Quinolone, macrolide, and tetracycline are used to treat disease caused by *Legionella* species, but resistance to these antibiotics is currently being reported steadily. Therefore, it is necessary to develop new antibiotics that have different mechanisms from conventional antibiotics.

There are 24 pairs of toxin-antitoxin system in *Legionella pneumophila*. The TA system is found in prokaryotes and consists of toxin proteins and a corresponding antitoxin that impairs toxin's

function. Previously, six types of TA system were reported, and types are classified based on the molecular types of toxin and antitoxin and the way they interact. In normal condition, the antitoxin combines with toxin to inhibit its toxicity, but in certain circumstances the antitoxin is degraded and the toxin is activated, resulting in cell-toxicity and inhibiting various processes in the cell. This induces the death of bacteria and hinders growth. We can find a new antibacterial mechanism for *Legionella pneumophila* by designing and developing antibiotic substances that can activate the toxin from TA complex.

Recently, new type of the TA system has been reported, and in that type, antitoxin impairs the function of toxin through the role of enzymes. According to the that paper, the HEPN-MNT protein in TA system belongs to type VII TA system, and antitoxin adenylylates its toxin using ATP as a substrate, and this mechanism of action inhibits the toxicity of toxin acting on RNase.

By studying the structure and function of lpg2921(MNT), lpg2920(HEPN), and HEPN-MNT complex, this paper tries to find the possibility of a new antibacterial mechanism for *Legionella pneumophila.*

Keywords : *Legionella pneumophila*, TA system, HEPN-MNT, lpg2921(HEPN), lpg2920(MNT).

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Contents

Ⅰ. Introduction

1.1. *Legionella pneumophila*

Legionella pneumophila is a pathogenic bacteria classified as gram-negative bacteria. One of the representative species is *Legionella pneumophila*. It is usually spread by breathing in aerosols that contain the bacteria, not by human-to-human contact. It causes an infectious disease called '*Legionellosis*', including *Legionnaires'* disease (a pneumonia-like illness), Pontiac fever (a non-pneumonia illness). About 20 percent of the world's bacterial pneumonia is known to be caused by *Legionella pneumophila*. Antibiotics such as quinolones, macrolides, and tetracyclines are used to treat '*Legionellosis*', but resistance to these antibiotics is currently being reported steadily. Therefore, it is necessary to develop new antibiotics that have a different mechanism from conventional antibiotics.

1.2. Toxin-Antitoxin system (TA system)

Toxin-antitoxin system is a genetic module that appears in prokaryotes and archaea, consisting of a toxin and a corresponding antitoxin. Toxin-antitoxin system is classified into different types based on the way toxin and antitoxin interact and what molecules they are. Each TA system pair belongs to its own type and family. Previously, six types of TA system were reported. In all types, toxin is a protein, and antitoxin appears in various molecular forms. In normal condition, the antitoxin combines with toxin to inhibit its toxicity, but in certain circumstances the antitoxin is

degraded and the toxin is activated, resulting in cell-toxicity and inhibiting various processes in the cell. This induces the death of bacteria and hinders growth. We can find a new antibacterial mechanism for *Legionella pneumophila* by designing and developing antibiotic substances that can activate the toxin from TA complex.

1.3. HEPN-MNT family in TA system

Various studies have been conducted on toxin-antitoxin system. However, related studies in *Legionella pneumophila* are limited. There are 24 pairs of toxin-antitoxin system in *Legionella pneumophila* and 3 pairs of them belong to the HEPN-MNT family. Although HEPN and MNT are the most common TA system families in prokaryotes, little research has been done on this family so far. The MNT family and accompanying HEPN family have been proposed to represent a novel, non-conventional TA system. In that type, antitoxin impairs the function of toxin through the role of enzymes, not by direct protein-protein interaction.

1.4. Objective of study

By understanding the protein structure and binding modes of toxin, antitoxin, and complex, we try to develop a new drug candidates that can attack key amino-acid residues which are important to TA complex formation. In this way, we can find a new antibacterial mechanism for *Legionella pneumophila.*

Ⅱ. Preparation for experiment

2.1. Materials

2.1.1. Reagents for experiment

Genomic DNA of *Legionella pneumophila* and primers for PCR were purchased from ATCC and BIONEER. XhoI and NdeI were purchased from New England Biolabs. pET28b(+) vector, DH5α, Rosetta (DE3) pLysS for cloning was purchased from Novagen. Luria Broth for cell culture is purchased from Novagen and Duchefa. Kanamycin were purchased from Sigma Aldrich. Cocktail protein inhibitors were purchased from Roche. Thrombin was purchased in Sigma Aldrich. Index and Wizard I-IV solution used in crystal screening were purchased from HAMPTON RESEARCH and Rigaku, respectively. Gel filtration marker for protein standard curve was purchased from Bio-RAD. RNase Alert kit for ribonuclease activity assay was purchased from Integrate DNA Technology (IDT). Primers for electrophoresis mobility shift assay (EMSA) were purchased from Bionics.

2.1.2. Instruments for experiment

Polymerase chin reaction (PCR) was performed by PCR system 9600 from Perkin Elmer. Cell lysis was performed by the sonic oscillator, Ultrasonic sonifier 450 produced by Branson ultrasonic Corporation. J2-MC and fraction collector were purchased from Bio-Rad. Centricon and centriprep were purchased from Millipore. His-tag affinity chromatography, Hi-Trap Q HP anion exchange chromatography column (5ml volume) were purchased from GE

healthcare. Specimens were observed in fast protein liquid chromatography (FPLC) from AKTA. Columns for size exclusion chromatography (for example, 75gl, 200gl, 75pg, and 200pg) were purchased from GE healthcare. Electrophoresis mobility shift assay were visualized by printgraph 2M (ATTO). Ribonuclease activity assay was conducted using SPECTRAmax GEMINI XS.

2.2. Methods

2.2.1. Gene cloning

PCR for amplification of genes encoding HEPN-MNT was conducted using *L. pneumophilla* genomic DNA as template strand. The genes encoding HEPN, MNT, and HEPN-MNT were all cut by NdeI and XhoI (restriction enzymes). pET28b(+) vector was also cut by these same restriction enzymes. They were combined into circular plasmids by ligation mix. Genes encoding pET28b(+) express proteins with hexa-histiding tag in N-terminal. We obtained recombinant plasmids for each of the lpg2921, lpg2920, and complex. The recombinant plasmids were transformed into DH5α competent cells and then transformed into Rosetta (DE3) pLysS competent cells for protein expressions. And then, they were kept in the freezer.

2.2.2. Over-expression and Purification

2.2.2.1. Protein over-expression

The recombinant gene was over-expressed in Rosetta (DE3) pLysS competent cells. These cells were grown in LB media with kanamycin for target cell selection at concentration 30mg/ml. The

temperature was 310K and the rotational speed was 180rpm. In OD_{600} is around 0.5, target proteins were induced by isopropyl β-D-1-thio-galacto-pyranoside (IPTG). About 4hours later, cells were harvested by centrifuge at 6,000rpm for 8minutes in 280K. After centrifuge, supernatant was discarded and only pellets are taken. The pellets were dissolved in A buffer (pH 7.9, 20mM Tris-HCl, 500mM NaCl) with 10% (v/v) glycerol. Cell lysis was conducted by sonicator. After this, the cell lysate was centrifuged at 18,500rpm for 1hour at 280K. Cell debris was discarded and only supernatant was taken.

In the case of complex proteins, low temperature incubation was carried out at 288K with a speed of 150rpm, and the cell harvest took place 20 hours after IPTG induction.

2.2.2.2. Protein purification

The supernatant was loaded into His-tag affinity column. Through this, target proteins stuck to the column. Subsequently, target proteins were eluted by concentration gradient of imidazole solutions. Only the fractions containing the target protein were collected, and concentration was carried out in buffer solution. (pH 8.0, 20mM Tris-HCl, 200mM NaCl). Then, protein solution was loaded into FPLC gel column. In case of HEPN-MNT complex, Ion exchange chromatography and thrombin-cutting were performed. Final protein solution was obtained through a series of purification processes. Concentration of final protein solution was set in the range of 0.2-0.4mM and stored in refrigerator at 193K.

2.2.3. Protein crystallization

Crystal screening was conducted by sitting drop vapor diffusion method using 96-well reservoirs. Final protein solution and crystal precipitants were reacted in different ratio. Index and Wizard I-IV were used as a crystal precipitants. They were stored at 293K. Formation or crystals took days to weeks and finally protein crystals were obtained. Hit crystals were optimized for X-ray data collection and sent to Pohang Accelerator Laboratory.

2.2.4. X-ray diffraction data collection
& 2.2.5. Protein structure determination

Protein crystals were sent to Pohang Accelerator Laboratory and X-ray diffraction data was collected. Raw data was processed using HKL-2000 program. Obtained protein structure was phased by single-wavelength anomalous dispersion. It was also refined by molecular replacement using the phase program in CCP4 suite of programs and Phenix. Manual model was conducted by Coot.

2.2.6. Protein standard curve

Proteins may have a different oligomeric state in crystal and solution states. Because protein exists in buffer solution, it is meaningful to know the oligomeric state of the protein in the solution. Based on a molecular weight of gel filteration markers from Bio-rad, the molecular weight and oligomeric state of the protein in the solution were predicted.

2.2.7. Ribonuclease activity assay

Sequence alignment was conducted using structure homology. RX_4HXY was a conserved motif between lpg2920(HEPN) and its structure homology, and these motifs had a RNase activity in common. $GSX_{10}DXD$ was a conserved motif between lpg2921(MNT) and its structure homology, and these motif also had RNase activity in common. Ribonuclease activity assay was performed to verify that this function was also present in our protein. Relative fluoroscence units (RFU) was detected on SPECTRAmax GEMINI XS spectrofluorometer. Absorption spectrum was measured at 490nm and emission spectrum was measured at 520nm.

2.2.8. Electrophoresis mobility shift assay (EMSA)

Electrophoresis mobility shift assay (EMSA) was conducted to determine if lpg2920(HEPN) and complex act as a transcription regulator by binding with a palindromic sequence in their promotor region. EMSA was conducted on lpg2920(HEPN) and HEPN-MNT complex. EMSA reaction was electrophoresed on a 0.5% agarose gel with 0.5X TBE and visualized using Printgraph 2M (ATTO). The promotor region and primers used in EMSA were as follows.

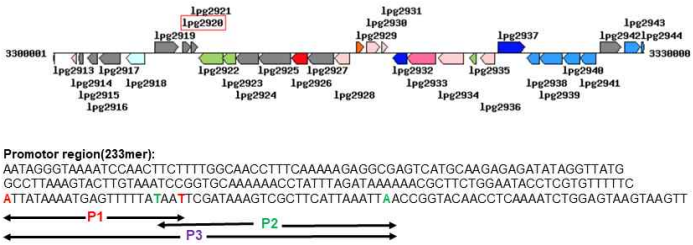


Fig. 1. Palindromic sequence of lpg2920 in promotor region

Ⅲ. Results

3.1. Protein over-expression and Purification

3.1.1. Protein over-expression

lpg2921 toxin belongs to MNT domain, and lpg2920 antitoxin belongs to HEPN domain. lpg2921(MNT) toxin, lpg2920(HEPN) antitoxin, and HEPN-MNT complex were expressed in Rosetta (DE3) pLysS (R_2P) competent cells and induced by IPTG.

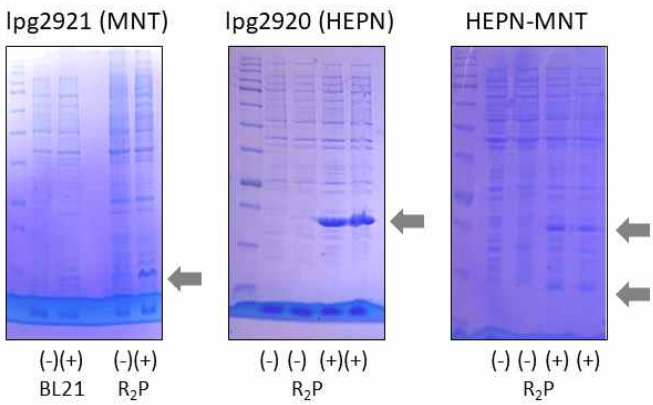


Fig. 2. Expression test

3.1.2. Protein purification

3.1.2.1. His-tag affinity chromatography

His-tag refers to several histidine attached to terminal of the recombinant protein and can be separately purified using the selective binding to metal ions. Histidine is usually well coupled with metal ions, such as Ni^{2+} and Fe^{2+}. When solutions containing recombinant proteins passes through a column filled with Ni^{2+} binding gel, only the recombinant protein joined by his-tag is attached to the column and the rest of the protein exits the

column. The target protein binding to Ni^{2+} is separated by the concentration gradient of the imidazole solution. Wash buffer was used at 25mM and elution buffer was used from 50mM.

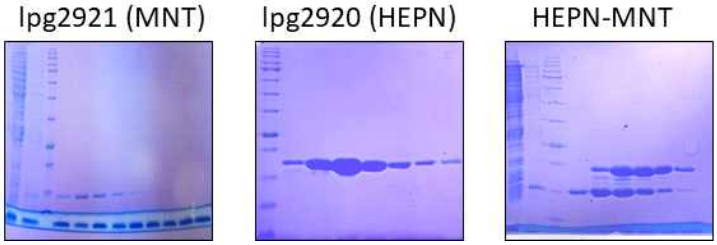


Fig. 3. Purification using His-tag affinity chromatography

3.1.2.2. Ion exchange chromatography (IEX)

Crystal formation of lpg2921(MNT) and lpg2920(HEPN) was successful. However, crystal formation of complex proteins failed. Thus, IEX and thrombin-cutting were performed for HEPN-MNT complex. IEX was performed for HEPN-MNT using Hi-Trap Q HP anion exchange chromatography column. Proteins elution were conducted by a concentration gradient of sodium chloride.

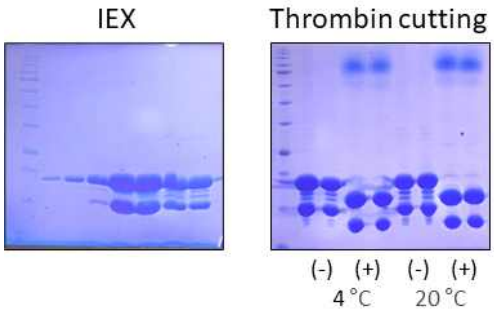


Fig. 4. Purification using ion exchange chromatography (left)

Fig. 5. His-tag cutting by thrombin (right)

3.1.2.3. His-tag cutting using thrombin

His-tag in N-terminal of the recombinant protein is a flexible structure that can interfere with regular packing of crystals. Thus, thrombin-cutting was carried out on HEPN-MNT complex protein.

3.1.2.4. Size exclusion chromatography (SEC)

To obtain pure protein and remove impurities, SEC was carried out using fast protein liquid chromatography (FPLC). FPLC is a liquid chromatographic device developed for purification or analysis of bio-materials such as proteins, peptides, and nucleic acids. Buffer condition for purification was pH 8.0, 20mM Tris-HCl, and 200mM NaCl. Purification of lpg2921(MNT) was carried out using a 75pg column, while purification of lpg2920(HEPN) and HEPN-MNT complex were carried out using 75gl column.

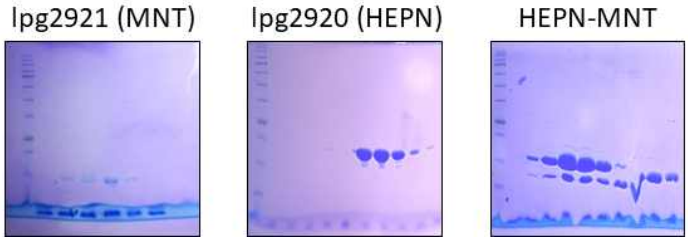


Fig. 6. Purification using FPLC gel column

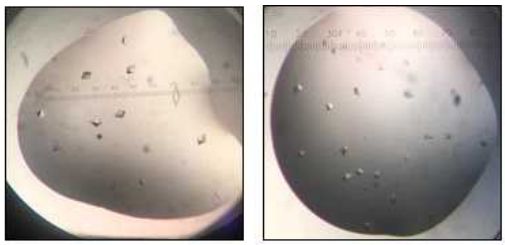
3.2. Protein crystallization & Protein structure analysis

3.2.1 Protein crystallization

Crystal screening was conducted by sitting drop vapor diffusion method using 96-well reservoirs. Final protein solution and crystal precipitants were reacted in volume ratio of (0.5μl : 0.5μl). Index and Wizard I-IV were used as a crystal precipitants.

① lpg2921(MNT)

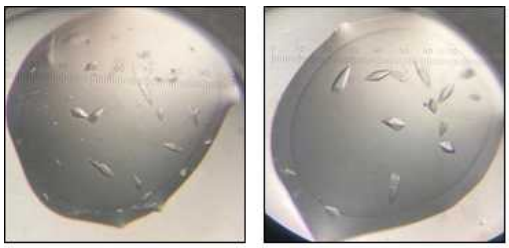
Fig. 7. Crystal of lpg2921(MNT)



- Precipitation Reagent : 20% (w/v) PEG 6000.
- Buffer : 100mM Citric acid / Sodium hydroxide pH 4.0
- Salt : 1000mM Lithium chloride.

② lpg2920(HEPN)

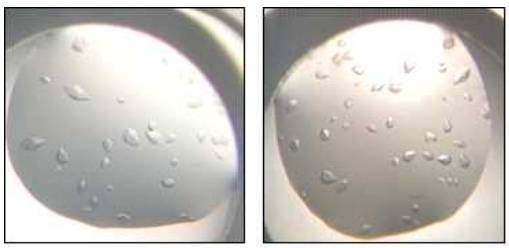
Fig. 8. Crystal of lpg2920(HEPN)



- Precipitation Reagent : 20% (w/v) PEG 3350.
- Buffer : 100mM Sodium citrate/ Citric acid.
- Salt : pH 4.0 200mM Sodium citrate tribasic.

③ HEPN-MNT

Fig. 9. Crystal of lpg2920-lpg2921 (HEPN-MNT)



- Precipitant : 20% w/v PEG monomethyl ether 2000.
- Buffer : 0.1M Tris pH 8.5
- Salt : 0.2M Trimethylamine N-oxide dihydrate.

3.2.2. Protein structure analysis

Based on X-ray diffraction data, structure of lpg2921(MNT) lpg2920(HEPN), and HEPN-MNT complex were calculated. Protein crystal structure analysis was performed using data processing programs. Through PyMOL, protein structure was visualized.

① lpg2921(MNT) toxin

lpg2921 appears in dimer consisting of two homogeneous MNT chains in crystal structure.

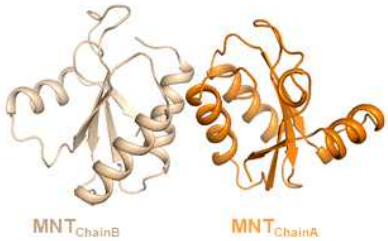


Fig. 10. Protein crystal structure of lpg2921

② lpg2920(HEPN) antitoxin

lpg2920 appears in octamer consisting of eight homogeneous HEPN chains in crystal structure.

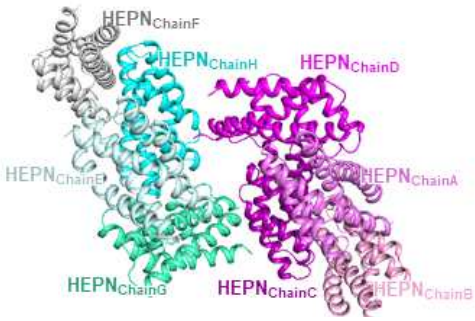


Fig. 11. Protein crystal structure of lpg2920

③ HEPN-MNT complex

Complex appears in decamer form consisting of eight HEPNs and two MNTs in crystal structure. One MNT and four HEPNs combine to form a pentamer, and these two pentamers form a decamer by combining through each MNT. In complex, MNT chain A binds to HEPN chain B, C, D, and E, respectively. Among them, the bonds A-B, A-D are the most important parts in complex formation.

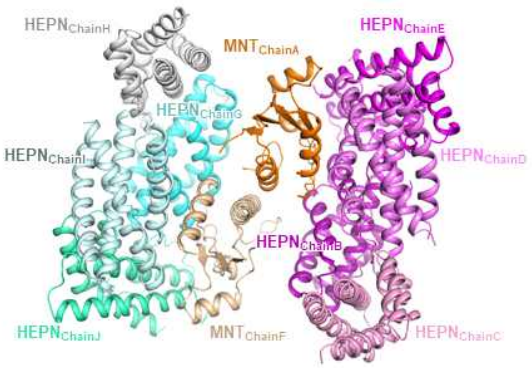


Fig. 12. Protein crystal structure of lpg2920-lpg2921 complex

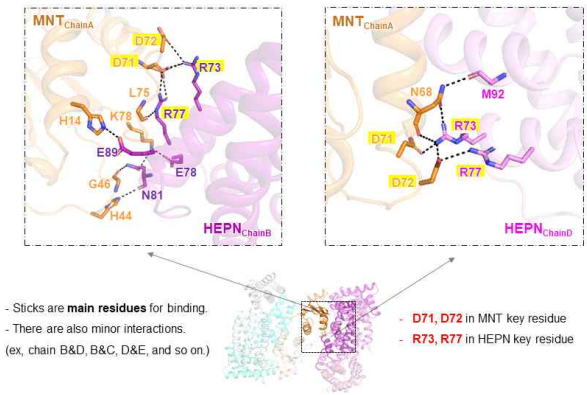


Fig. 13. Major hydrophilic interaction of lpg2920-lpg2921 complex

In addition, D71, D72 in MNT and R73, R77 in HEPN are common in these bonds. Therefore, these amino acid residues are key residue in complex formation.

3.3. Protein standard curve

To predict the organic state of proteins in solution, a protein standard curve was obtained using the Superdex 200gl column. The flow rate was 0.5ml/min and 20mM tris, 200mM NaCl, and pH 8.0 buffer was used for mobile phase. In the solution, it was confirmed that HEPN-MNT complex, lpg2921(MNT) toxin, and lpg2920(HEPN) antitoxin were each taking an oligomeric state of pentamer, dimer, and tetramer.

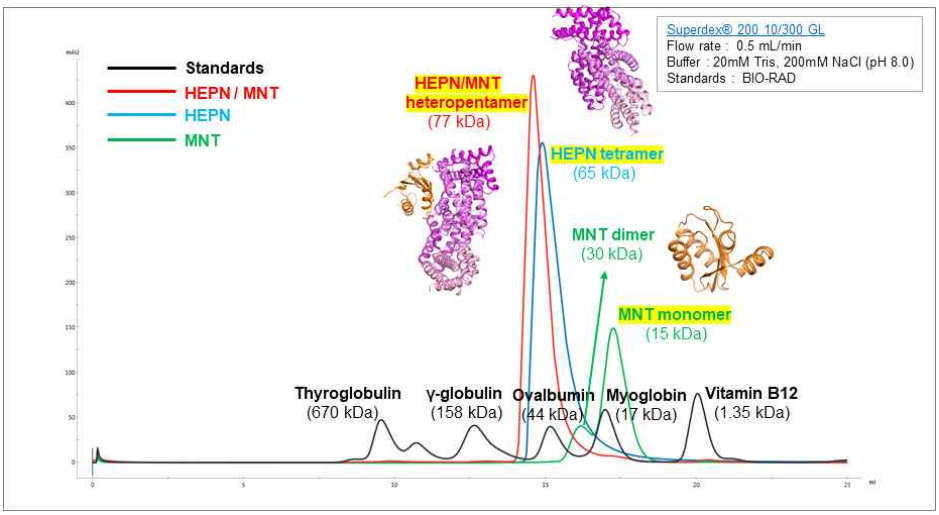


Fig. 14. Protein standard curve

3.4. Ribonuclease activity assay

① lpg2921(MNT)

It is common for toxin to exhibit RNase activity in the TA system. In lpg2921(MNT) and its structure homology, RX_4HXY motif was conserved, and RNase activity was predicted to occur in this motif. Therefore, Ribonuclease activity assay was conducted, lpg2921(MNT) showed concentration-dependent RNase activity.

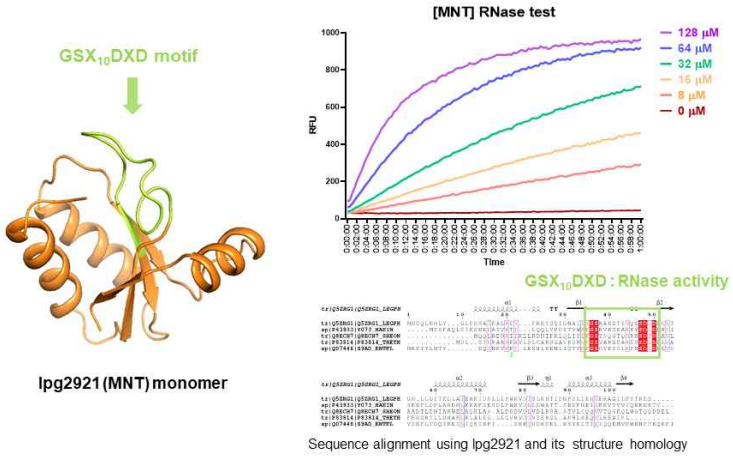


Fig. 15. Ribonulease acitivity assay of lpg2921 (MNT)

② lpg2920(HEPN)

In lpg2920(HEPN) and its structure homology, $GSX_{10}DXD$ motif was conserved, and this motif was also expected to exhibit RNase activity. Therefore, Ribonuclease activity assay was conducted for lpg2920(HEPN) antitoxin. It also showed concentration-dependent RNase activity. It is rare for antitoxin to show RNase activity in TA system. So this was a very interesting result.

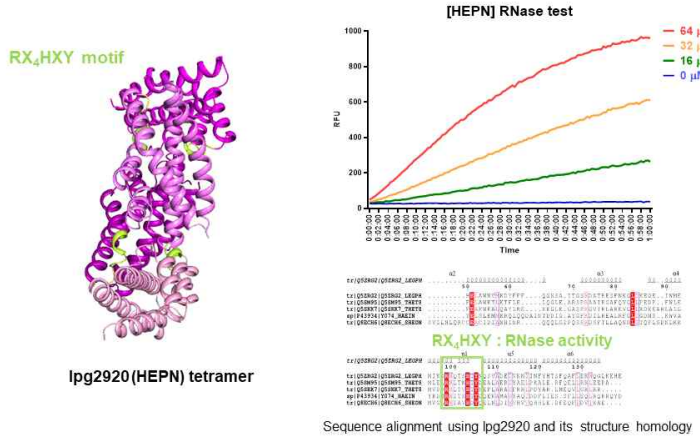


Fig. 16. Ribonulease acitivity assay of lpg2920 (HEPN)

③ HEPN-MNT complex

RNase activity was shown when toxin and antitoxin were present alone, but when they existed as a complex, RNase activity disappeared. Through this, we confirmed that antitoxin is inhibiting toxin's function in TA complex.

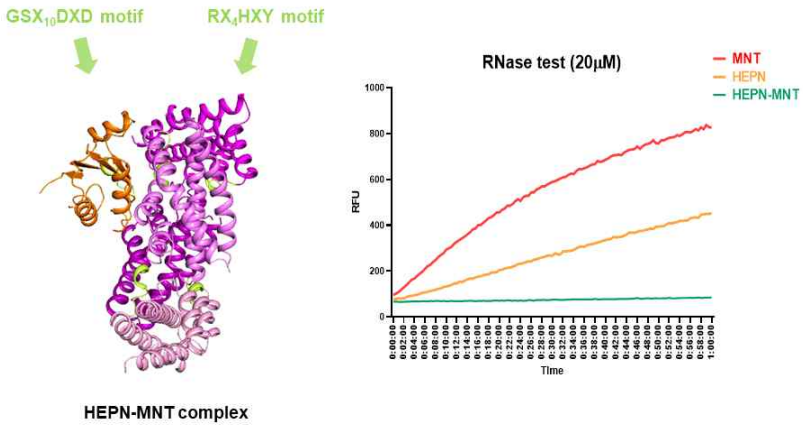


Fig. 17. Ribonulease acitivity assay of lpg2920-lpg2921 (HEPN-MNT)

The disappearance of toxicity on complex is a natural result from a perspective of toxin antitoxin system, but it can be analyzed structurally. HEPN has a side that MNT binds and a side that does not bind. The two interfaces look similar in the picture.

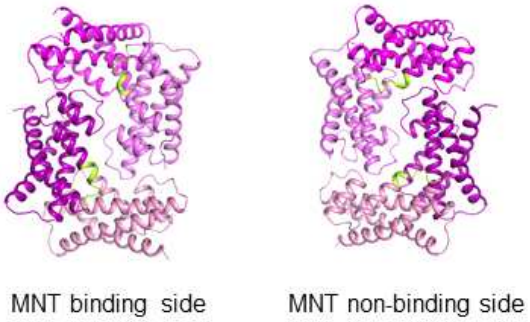


Fig. 18. MNT binding side and MNT non-binding side in lpg2920 (HEPN)

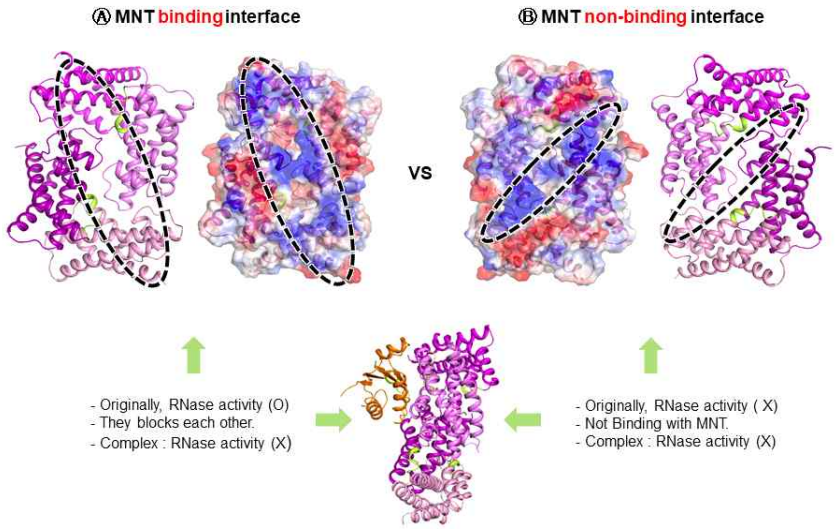


Fig. 19. Surface charge and binding interface of lpg2920 (HEPN)

However, from the surface charge perspective, the difference is seen. Blue means (+) charge and red means (-) charge. The interface that MNT binds is long and wide (+) charge valley, and the interface that MNT does not bind, forms a thin and narrow (+) valley. RNA gets access to proteins through this (+) valley.

In the interface that MNT binds, it passes through RNase active site, and in the interface where MNT does not bind, it passes the RNase active site. Interface that MNT binds originally has RNase activity, but RNase activity does not appear because toxin and antioxin combine to prevent each other's RNase active sites. Interface that MNT does not bind does not originally indicate RNase activity, so RNase activity does not appear in this area, even though it is exposed externally on complex.

3.5. Electrophoresis mobility shift assay (EMSA)

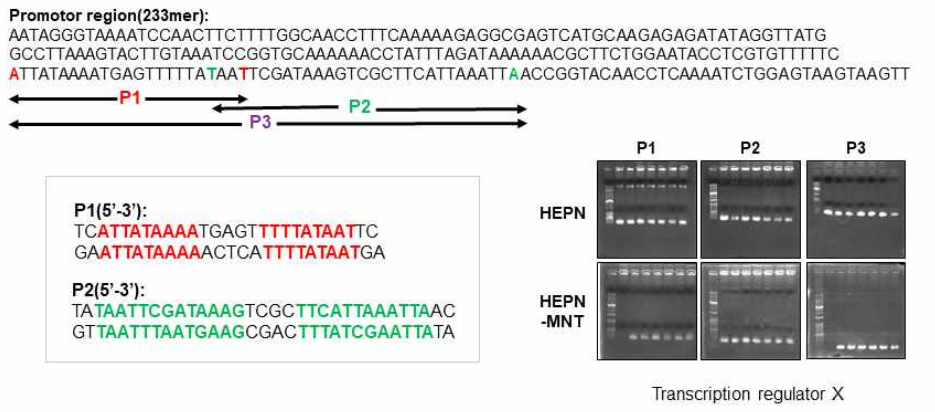


Fig. 20. EMSA result of HEPN and HPEN-MNT

Antitoxins in the TA system often act as a transcription regulator when they exist alone or as a complex, by binding them to the palindromic sequence in their promoter regions. To find out that these functions were also present in our proteins, EMSA was conducted for antitoxin and complex. As a result, there were no significant band shifts and the function of a transcription regulator did not appear in our protein. In addition, like ribonuclease activity assay, it was once again confirmed that the antitoxin showed a different appearance from antitoxins in other TA systems.

Ⅳ. Discussion

Although HEPN-MNT family was the most common TA system in prokaryotes and archaea and has been previously predicted as a type II TA system, its function and mechanism of action remained unclear. However, there may be a reason for this large number of HEPN-MNT to be found in prokaryotes.

Recently, new types of TA system has been reported. In type VI TA system, when complex is formed, antitoxin facilitates the degradation of the toxin. Also, several novel TA systems have been reported in which the antitoxin functions as enzymes, expanding the ways antitoxins neutralize their toxin. They are called the Type VII TA system and show a unique mechanism of action. For example, In Hha/TomB, antitoxin oxidizes Cys of the toxin. In ToxSAS/antiToxSAS, antitoxin degrades the product of the toxin. In TglT/TakA, antitoxin phosphorylates Ser of the toxin. In HEPN/MNT, antitoxin consecutively poly adenylylate the toxin using ATP as a substrate. lpg2921(MNT) and lpg2920(HEPN) are also likely to belong to this new TA system, given that they play the role as enzyme, respectively.

This structure-function study on lpg2920-lpg2921 HEPN-MNT identify structure and function of HEPN-MNT in pathogenic bacteria for the first time. Also, it revealed the structure and function of TA system in *Legionella pneumophila* for the first time. In this paper, we present the possibility of a new antibacterial mechanism for Legionella bacteria by revealing the structure and function of the lpg2921, lpg2920, and complex proteins.

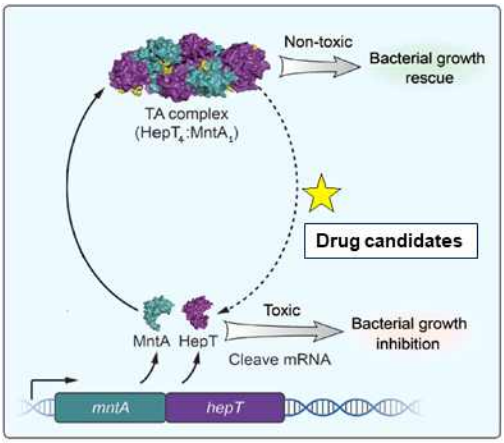


Fig. 21. Neutralization mechanism of the HEPN-MNT TA system

When they exist independently, each has an RNase activity and do not exhibit RNase activity in complex. Therefore, a substance that can activate complex with toxin and antitoxin can be used as a strong antibiotic mechanism by causing RNase dual action.

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Ⅵ. Abstract in Korean (국문초록)

레지오넬라균은 그람 음성균으로 분류되는 병원성 균으로서 대표적인 종으로는 *Legionella pneumophila*가 있다. 전 세계 세균성 폐렴의 약 20%가 레지오넬라균에 의해 발생된한다고 알려져 있다. 레지오넬라균의 치료에는 quinolone, macrolide, tetracycline 계열 항생제들이 사용되고 있으나, 현재 해당 항생제들에 대한 내성이 꾸준히 보고되고 있다. 따라서, 기존 항생제와는 다른 새로운 메커니즘의 항생제 개발이 필요한 실정이다.

*Legionella pneumophila*에는 24쌍의 toxin-antitoxin system (TA system)이 있다. TA system은 원핵생물에서 발견되며 toxin과 그들의 기능을 저해하는 antitoxin으로 구성된다. 기존에는 6가지 type의 TA system이 보고되었으며, toxin과 antitoxin의 분자적 종류와 그들이 상호작용하는 방식을 기준으로 type이 구분된다. 평소에는 antitoxin이 toxin과 결합하여 그 독성을 저해하고 있지만, 특정 상황에서는 antitoxin이 분해되고 toxin이 활성화되어 독성이 나타나고 세포 내 다양한 과정을 저해하게 된다. 이는 결과적으로 박테리아의 사멸 또는 성장저해를 일으키게 된다. 우리는 TA complex로부터 toxin을 활성화하는 항생물질을 설계 및 개발함으로써 레지오넬라균에 대한 새로운 항균 메커니즘을 찾아낼 수 있다.

HEPN-MNT계열은 원핵생물에서 가장 흔하게 나타나는 계열의 TA system임에도 불구하고, 그 기능과 저해 메커니즘에 대해 명확하게 밝혀지지 않은 상태였다. 그러나, 최근 antitoxin이 enzyme 작용을 통해 toxin의 기능을 저해하는 새로운 type의 TA system이 보고되었으며 'Nuclear Acids Research'에 수록된 'Novel polyadenylylation-dependent neutralization mechanism of the HEPN/MNT toxin/antitoxin system'에 따르면 고세균 유래 HEPN-MNT 계열 TA system이 type Ⅶ에 속하며, antitoxin은 ATP를 기질로 하여 toxin을 adenylation하고, 이를 통해 RNase 작용을 하는 toxin의 독성을 저해한다는 사실이 보고되었다. 본 논문에서는 레지오넬라균 유래 HEPN-MNT TA system에 해당하는 lpg2921, lpg2920, complex 단백질의 구조와 기능을 밝힘으로써, 레지오넬라균에 대한 새로운 항균 메커니즘의 가능성을 제시하고자 한다.