藥學碩士學位論文

Structural study of Rv2757c (Endoribonuclease) from *Mycobacterium tuberculosis* H37Rv

Mycobacterium tuberculosis H37Rv 에서 유래한

Rv2757c 단백질의 구조연구

2015 年 **2** 月

서울대학교 대학원

약학과 물리약학전공

박 형 준

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指導敎授 李 奉 振
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2015 年 **2** 月

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Abstract

Structural study of Rv2757c (Endoribonuclease) from *Mycobacterium tuberculosis* H37Rv

Park Hyong-Jhun, **physical pharmacy**, **the graduate school,**

Seoul national university

Mycobacterium tuberculosis is one of the oldest pathogenic bacterial species ever human being experienced. In 2013, there were 9.0 million new TB cases and 1.5 million TB deaths. Among these casualties, 210,000 deaths were estimated to be from multi drug-resistant TB. There are some antibiotics for TB; however these antibiotics have shown the emergence of drug-resistant *M.tb*. SBDD (Structure-Based Drug Discovery) enables a novel drug to be developed more effectively based on the structural information of the target protein. Toxin-Antitoxin system was selected to develop a new antibiotics for TB. TA systems were classified into five classes based on the genetic architecture and the nature of regulation of TA systems. Type II TA systems consist of an antitoxin protein that sticks to and neutralizes the toxin's activity by direct binding interaction. The Rv2757c toxin composed of 138 amino acids has been estimated to a VapC toxin which has a ribonuclease activity. VapBC family occupies 20 of 38 functional TA systems of *M.tb*. VapC toxins has highly conserved acidic amino acids which are

thought to be putative catalytic residues in PIN domain. To determine the structure of the Rv2757c toxin, the gene of the Rv2757c toxin was cloned in pCold vector. Consequentially, the protein was over-expressed in *E.coli* cells. The proteins were purified with IMAC affinity chromatography and SEC. The purified protein was used in formation of crystals using crystal-screening kits. The well-made crystals appeared in 2-3 days in optimized reservoir solution consisting of 20% (w/v) PEG-8000, 0.1 M CHES/sodium hydroxide pH 9.5. X-ray diffraction data was collected using synchrotron radiation on ADSC Q315r detector at beamline PAL-5C (SBII) (Pohang, South Korea) at λ = 0.97944. Crystal of the Rv2757c toxin belonged to the monoclinic space group C2 (C121), with unit cell parameters of a=61.465 Å, b=44.813 Å, c=57.486 Å, and α=γ=90, β=99.87°. The crystal structure of the Rv2757c toxin shows that it consists of seven α-helices, five β-strands and one 3_{10}-helix, and the sequence of β - α - α - β - α - α - β - α - 3_{10} - α - β - α - β helices and strands. Four mutants of the Rv2757c were purified to determine if the conserved residues are necessary in ribonuclease activity of the Rv2757c toxin. The wild type and four mutants of the Rv2757c toxin were used in agarose gel electrophoresis with synthesized mRNA and fluorescence spectroscopy with synthesized mRNA and fluorescent spectroscopy with fluorescent-labeled RNA substrate. Consequently, the Rv2757c toxin protein showed ribonuclease activity *in vitro*, and two residues (D97, D115) of the Rv2757c toxin were predicted to be in part of ribonuclease activity.

Key words: *Mycobacterium tuberculosis*, SBDD (Structure-Based Drug Discovery), Rv2757c, X-ray crystallography, VapC toxin, fluorescent spectroscopy

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국문초록

Abbreviations

3D	Three dimensional
CHES	2-(Cyclohexylamino)ethanesulfonic acid
E. coli	Escherichia coli
EDTA	Ethylenediaminetetraacetic acid
M.tb	Mycobacterium tuberculosis
M.tuberculosis	Mycobacterium tuberculosis
IPTG	Isopropyl-β-D-thiogalacopyranoside
LB	Luria Bertani
OD	Optical Density
PAGE	PolyAcrylmide Gel Electrophoresis
PCR	Polymerase Chain Reaction
PEG	PolyEthyleneGlycol
RNase	Ribonuclease
SEC	Size Exclusion Chromatography

I. Introduction

1.1. Concept of SBDD

SBDD equates to the abbreviation of Structure Based Drug Discovery. SBDD is to make a new drug based on the structure of the target molecule.

Knowledge of the 3D details of a given target protein can greatly promote both the identification of new lead structures and the optimization of their binding, based on complementarity between ligand and receptor. Since drug discovery depends heavily on the identification of novel compound, the potential for generating lead compound without screening techniques and a reduced amount of time essential for lead optimization can render significant savings in time and money in drug development.

Several drugs were made by SBDD process. For example, Viagra®, Gleevec®, Tamiflu®, Pradaxa® were developed targeting PDE-5, BCR-ABL kinase, Neuraminidase, thrombin, based on SBDD, respectively.

1.2. *Mycobacterium tuberculosis*

Mycobacterium tuberculosis is one of the oldest pathogenic bacterial species ever human being experienced. [Issar Smith., 2003] In 2013, there were 9.0

million new TB cases and 1.5 million TB deaths. Among these deaths, 210,000 deaths were estimated to be from multi drug-resistant TB.

The feature of *M.tb* that exhibits the most significant obstacle to developing treatment-shortening therapies for tuberculosis is its ability to persist in the face of the threats imposed by host immunity and bactericidal drug action. Thus, understanding the physiology of the persistent states in *M.tb* can be one of the most important areas of recent mycobacterial investigation.

Much importance has been presented to define the physiology of non-replicating persister cells of *M.tb* formed under the conditions of nutritional, hypoxic, and acidic stress thought to be encountered during infection.

This has led to the identification of pathways that are needed to maintain mycobacterial survival under such conditions and whose components are being thought to be novel drug targets.

1.3. Toxin-Antitoxin system

The toxin–antitoxin (TA) systems were discovered in the 1980s as plasmid genes that guarantee inheritance of a plasmid in daughter cells (Ogura & Hiraga, 1983; Gerdes et al., 1986). Homologues of TA plasmid system modules were later found in the chromosomes of most bacteria, and their roles in bacterial cells have been intensively investigated.

TA systems are ubiquitous in prokaryotic genomes and have been proposed to play several roles in cellular functions. Prokaryotic TA systems code for two components, a stable ‘‘toxin’’ (a protein) that affect important cellular functions such as translation, replication and cell-wall synthesis, cell growth and an unstable ‘‘antitoxin’’ (either RNA or protein) that regulates toxin activity. The genetic architecture and the nature of regulation of TA systems showed that they can be divided into five classes. Type I and III TA systems encode small RNA antitoxins that interact with the toxins at the translational levels (antisense RNA) or posttranslational levels (direct toxin binding), respectively. Type II TA systems consist of an antitoxin protein that sticks to and neutralizes the toxin’s activity by direct binding interaction. In type IV TA systems, the antitoxins protect the toxin target molecule instead of inhibiting the toxin directly (Masuda et al., 2012). Type V antitoxins are site-specific endoribonucleases that inhibit toxin expression by cleavage of toxin-encoding mRNAs (Wang et al., 2012).

The most common mechanism of TA system toxicity is mediated through mRNA cleavage, resulting in translation inhibition.

In normal cells, toxin and antitoxin proteins form stable complexes to inhibit the function of toxins. (Yamaguchi Y. and Inouye M., 2009) However, antitoxins are degraded by stress-induced protease under several stressful environments such as hypoxia, nutritional starvation, and treatment of antibiotics.

1.4. VapBC family and endoribonuclease activity of VapC

The genome of *Mycobacterium tuberculosis* H37Rv contains MazEF, RelBE, ParDE, HigBA and VapBC families of type II TA system.

The largest family of TA systems in *M.tb* is VapBC (Virulence associated protein) family. VapBC family occupies 20 of 38 functional TA systems of *M.tb*. VapC toxins contain PIN domains (homologous to PilT N-terminal domain), a motif thought to be involved in ribonuclease activity and have been demonstrated to block translation by mRNA cleavage. Even though sequence similarity is low over most of PIN domains, sequence alignments have indicated that active site residues are highly conserved. These residues were first predicted *in silico* to have a nuclease activity which was then confirmed *in vitro*. Most of the VapC toxins were tested so far, VapC1, VapC2, VapC5, VapC11, VapC20, and VapC29 from *M. tuberculosis*, exhibit a ribonuclease activity in vitro.

Several VapBC of *M.tb* were known as being induced in response to relevant stress environments faced during the infection process. This incorporates hypoxia (VapBC7, VapBC15, and VapBC25) and in IFN-γ-stimulated murine bone marrow-derived macrophages (VapBC3, VapBC11, and VapBC47). In addition, VapBC3, VapBC31, and VapBC49 were shown to be the most up-regulated in drug-tolerant *M.tb* among other TA systems, suggesting a possible factors in formation of persistence.

1.5. Purpose of the study

The purpose of the study is to determine the tertiary structure of proteins from *Mycobacterium tuberculosis* H37Rv, to demonstrate the function of the hypothetical proteins through structural study, and to discover novel pharmacophore of *M.tb* antibiotics. To achieve the goal, fourteen *M.tb* proteins were selected and cloned through the known genome sequence. They were produced by *E.coli* expression system and the expressed proteins were carried out purification by chromatography. Solubility test was carried out on the expressed proteins to optimize the best soluble condition of the proteins. Among the several proteins, Crystal screening step was carried out and we could get well-made crystals of the Rv2757c toxin. Finally, three-dimensional structure of the Rv2757c toxin was determined by X-ray crystallography.

II. Materials and methods

2.1. Materials

2.1.1. Reagents

The whole genome of *Mycobacterium tuberculosis* H37Rv was bought from BIONEER. Sequencing service was from COSMOGENETECH. PCR premix kits were purchased from INTRON biotechnology, Inc. pCold I vector, T4 DNA ligase, T4 DNA ligase buffer were purchased from Takara Inc. Bovine serum albumin, restriction endonucleases, and buffer 4 were purchased from New England Biolabs (NEB). IPTG was purchased from CALBIOCHEM. Ampicillin was bought from BIOSESANG. Ni^{2+}-agarose IMAC column (His-bind Resin) was purchased from GE Healthcare Inc. (USA). EDTA was purchased from Biobasic Inc. (Markham, ON, Canada). EZchange site-directed mutagenesis kit was purchased from Enzynomics Inc. MEGAshortscript kit, Megaclear kit, RNase Alert kit, and RNaseZap were bought from Life technology Inc. All reagents except for the list stated above were purchased from BIOSESANG.

2.1.2. Apparatus

PCR reaction was carried out by Perkin-Elmer PCR system 9600 (Perkin-Elmer, U.S.A.). Cell lysis was performed by the sonic oscillator, sonifier 450 produced by Branson Ultrasonic Corporation (Connecticut, U.S.A.). J2-MC and the fraction collector were purchased from Bio-Rad Laboratories Inc. (California, U.S.A.). Centricon, Centriprep were purchased from Millipore Corporation (Massachusetts, U.S.A.). Specimens were observed in Fast Protein Liquid Chromatography (FPLC) (AKTA, U.S.A.). Size exclusion chromatography was performed by Superdex 75 10/300 GL and (GE Healthcare, U.S.A.). Fluorescence spectroscopy was performed with fluorimeter Gemini XS (Molecular device, U.S.A).

2.2. Methods

2.2.1. Cloning of target protein

Recombinant plasmids for the Rv2757c toxin was constructed using pCold I vector system to obtain large quantity of the protein. The Rv2757c gene was amplified by polymerase chain reaction using the whole genome of *Mycobacterium tuberculosis* H37Rv strain as template. Sequence of the forward primer used in PCR amplification includes Nde I-recognition site (CAT ATG), while backward primer includes Xho I-recognition site (CTC GAG). The

amplified DNA fragment was inserted into the Nde I / Xho I-digested pCold I expression vector (Takara). Then it was transformed to competent cell of *E.coli* DH5α. After validation of target protein's sequence, the recombinant plasmid was transformed to competent cell of *E.coli* C41 (DE3) to perform the over expression test with IPTG.

2.2.2. Over-expression and purification

The *E.Coli* C41(DE3) cells containing the specific plasmid of *M.tuberculosis* protein (Rv2757c) stored at -80℃ were cultivated overnight in 40 ml of LB media containing ampicillin (50 g/ml) at 37℃. The cells cultivated in the seed culture fluid were inoculated into the newly autoclaved LB media (800 ml*2). They were incubated at 37℃, 180 rpm shaking condition until the value of Optical Density (O.D) in wavelength of 600 nm becomes 0.5~0.6. After cooling-down, the over-expression of the protein was induced by IPTG (0.5 μg/ml at final concentration). The cells were incubated overnight at 15℃, 180 rpm shaking condition. The cells were harvested by centrifugation (Beckman J2-MC) at 4℃ and 8000rpm for 10 minutes. The pellet of the cell was suspended in 40 ml of lysis buffer (50 mM Tris-HCl, 500mM NaCl, pH 8 and 5% glycerol) and homogenized by sonication (40% amplitude, pulse on 2 seconds, pulse off 4 seconds, total pulse on 30minute in ice). The entire lysate was centrifuged at

15000rpm for 1hr at 4℃. The cell debris was discarded and the supernatant was loaded into IMAC column (2.5*20 cm) previously equilibrated by washing buffer (50 mM Tris-HCl, 500 mM NaCl, pH 8) The column was washed with the same buffer (washing buffer) by gravity flow. The target protein sticking to the resin was eluted with the method of stepwise imidazole gradient (20, 50, 100, 200, 300, 500 mM of imidazole). The purified fractions containing target protein were pooled and concentrated. The concentrated solution was passed through Superdex 75 SEC column equilibrated by the same washing buffer. The eluted solution was diluted into the dialysis buffer (20 mM sodium phosphate, 100 mM NaCl, pH 6.5). The solution was then concentrated until the concentration of target protein becomes approximately 0.2 mM. All the purified fractions were analyzed by SDS-PAGE electrophoresis and concentrated by ultrafiltration (Centricon, Millipore Corp.)

2.2.3. Crystallization

Crystallization conditions were searched by the sitting drop vapor diffusion method at 20℃ using screening kits from Hampton Research Inc. (Crystal Screen I, II, Index I, II) and from Emerald Biosystems Inc. (Wizard I, II, III, and IV). The crystallization drops were made by mixing the protein solution and the reservoir solution in one-on-one ratio. The well-made crystals appeared in

2-3 days in optimized reservoir solution consisting of 20% (w/v) PEG-8000, 0.1 M CHES/sodium hydroxide pH 9.5. The crystal was vitrified using the cryoprotectant solution consisting of the reservoir solution supplemented with 25 % (v/v) glycerol. Crystals were soaked in the cryoprotectant solution for a few seconds before being frozen in liquid nitrogen.

2.2.4. X-ray data collection and structure determination

X-ray diffraction data was collected using synchrotron radiation on ADSC Q315r detector at beamline PAL-5C (SBII) (Pohang, South Korea) at λ = 0.97944. Crystal of the Rv2757c toxin belonged to the monoclinic space group C2 (C121), with unit cell parameters of a=61.465 Å, b=44.813 Å, c=57.486 Å, and α=γ=90, β=99.87°. Raw data was processed and scaled using HKL2000 program package (Otwinowski and Minor, 2002). The structure was determined by molecular replacement using the phase program in CCP4 suite of programs and Phenix (Adams et *al*., 2010) using *M.tuberculosis* Rv0301 (PDB ID: 3H87) as search model. Coot (Emsley and Cowtan, 2004) was used for manual model.

2.2.5. Homology search for the Rv2757c toxin

Dali program was used to understand the relationship between 3D structure and function. Dali program is a web server for comparison of protein

structures. Dali program compares coordinates of a query protein structure against 3D structure of other proteins in Protein Data Bank (PDB). The database of the Dali program allows searching pre-computed structurally similar neighborhoods. Comparing protein structures in 3D can detect biologically interesting similarities that cannot be revealed by comparing sequences (Liisa Holm *et al*., 2010).

2.2.6. Ribonuclease activity assay

The Rv2757c toxin has been estimated to be a VapC toxin which has a ribonuclease activity. As statements from introduction, VapC toxins has highly conserved acidic amino acids which are thought to be putative catalytic residues in PIN domain. Based on sequence alignment of the Rv2757c toxin and the Rv0301 toxin by BLAST (Basic Local Alignment Search Tool), it was known that the Rv2757c toxin has four highly conserved amino acids (Asp 8, Glu 42, Asp 97, and Asp 115).

To verify if the residues are really critical to nuclease activity of the Rv2757c toxin, four mutants which have each putative catalytic residues mutated to alanine were cloned using EZchange (Enzynomics) followed by over-expression and purification as wild type was.

Using T7 MEGAshortscript kit, mRNA fragment coding the Rv2757c toxin itself was synthesized. It was used as the substrate for ribonuclease activity assay visualized on 2.5% agarose gel electrophoresis.

Fluorescent-labeled RNA substrate of unknown sequence from RNase Alert kit was incubated with proteins for about 3 hours. In this assay, fluorescence is detected when the substrate was cleaved by nuclease. This method can assess ribonuclease activity of enzymes using fluorimeter. This assay was used in comparison of mutants' ribonuclease activity and verifying metal ion-dependence of the Rv2757c toxin's ribonuclease activity. All pipette tips and equipment are wiped with RNase Zap to prevent ribonuclease contamination followed by sterilization step.

Ⅲ. Results

3.1. Over-expression and purification

3.1.1 Over-expression and solubility test

The Rv2757c toxin is a protein whose number of amino acid is 138 and the molecular weight is 15,773 Da. The theoretical pI value is 6.65. Rv2757c was cloned to pCold I vector which has a hexa-histidine tag on N-terminus of inserted protein. Competent cell of *E.coli* C41 (DE3) was chosen to over-express Rv2757c protein. The cells were incubated in LB medium overnight at 15℃ and 180 rpm after induction by adding IPTG. The result of expression test is shown in Figure 1.

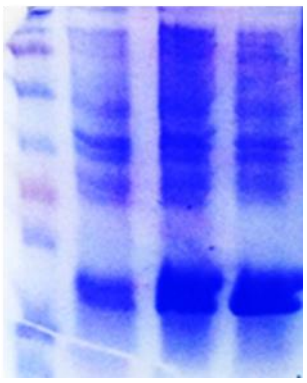

Figure 1. Expression of Rv2757c

After sonication on cell pellet, solubility test of the Rv2757c toxin was carried out. A few proteins were in soluble fraction in LB media. (Figure 2.)

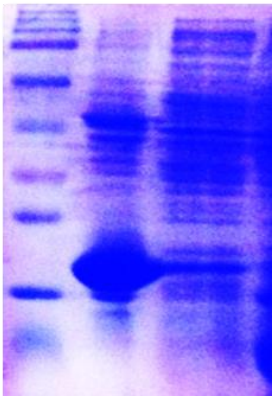

Figure 2. Solubility test of Rv2757c

3.1.2 Purification

Result of purification using IMAC column is given in Figure 3. The Rv2757c toxin was purified with high purity.

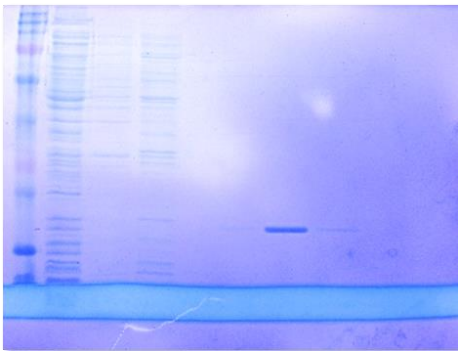

Figure 3. The result of purification using IMAC column

The purified fractions were concentrated followed by size-exclusion chromatography using Superdex 75 10/300 GL column. (Figure 4.)

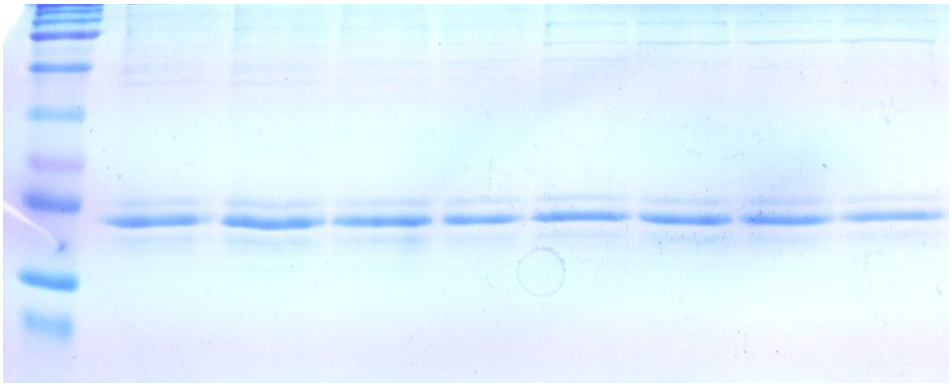

Figure 4. The result of purification using SEC

After purification, protein was dialyzed into dialysis buffer followed by concentration to 0.2mM, approximately.

3.2 Crystallization

The crystals of the Rv2757c toxin made by hanging-drop vapor diffusion are shown in figure 5. Multiple attempts resulted in poorly diffracting crystals with low reproducibility, but only crystals which were formed in 20% (w/v) PEG-8000, 0.1 M CHES/sodium hydroxide pH 9.5 could diffract X-ray well. Crystals of the Rv2757c toxin were in thick and flat needle shape. (Figure 5.)

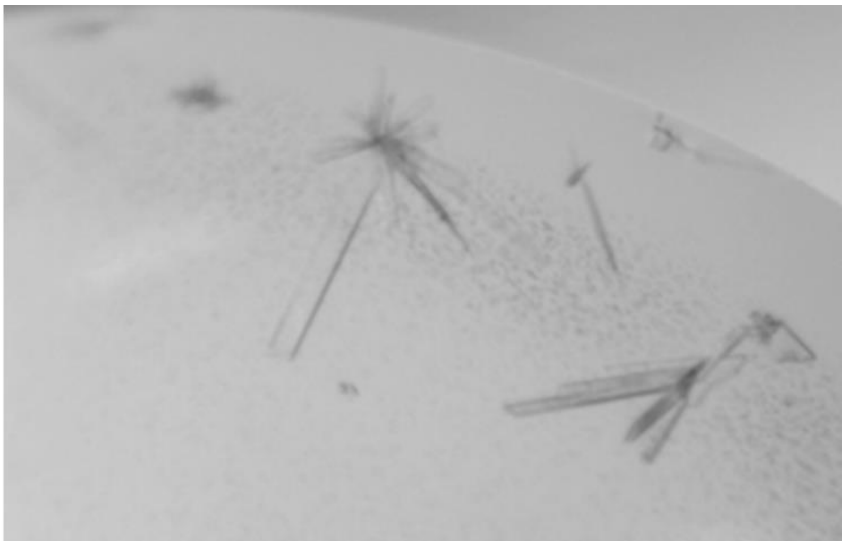

Figure 5. Crystals of Rv2757c

3.3 Crystal structure of Rv2757c

Figure 6. shows the crystal structure of the Rv2757c toxin consisting of seven α-helices, five β-strands and one 3_{10}-helix, and the sequence of β - α - α - β - α - α - β - α - 3_{10} - α - β - α - β helices and strands. Table 1 summarizes the data collection statistics.

Table 1. Data collection statistics

	Rv2757c
Data collection	
Beamline	PAL-5C (SBII)
Wavelength (Å)	0.97944
Space group	C2
Cell dimension (Å)	
	a = 61.47, b = 44.81, c = 57.49
	α = γ = 90.00°, β = 99.87°
Resolution (Å)	36.05–1.52 (1.55–1.52)[b]
R_{merge}[a] (%)	5.7 (22.5)[b]
$\langle I \rangle / \langle \sigma(I) \rangle$	53.30 (10.5)[b]
Redundancy[c]	5.9 (5.9)[b]
Completeness (%)	99.0 (99.0)[b]
Unique reflections	23634
Refinement	
R_{work}[d] (%)	18.9
R_{free}[e] (%)	20.4
No. Atoms	
Proteins	1120
Water	85
RMSD[f]	
Bond lengths (Å)	0.009
Bond angles (°)	1.318
Ramachandran plot (%)	
Preferred region	99.24
Allowed region	0.76
Outliers	0

a $\sum| I_j - \langle I \rangle | / \sum I$

b The values in parentheses indicate the highest resolution shell

c N_{obs} / N_{unique}

d $\sum_{hkl} | | F_{obs} | k | F_{calc} | | / \sum_{hkl} | F_{obs} |$

e R_{free} was calculated same way as R_{work}, but with the 5% of the reflections excluded from the refinement.

f Root mean square deviation (RMSD) was calculated with REFMAC

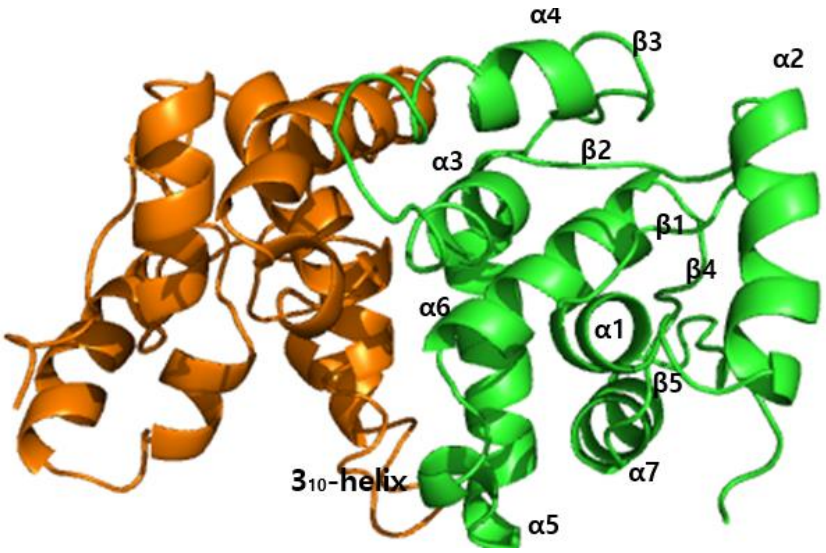

Figure 6. Crystal structure of Rv2757c dimer

The Rv2757c toxin molecule forms a tightly packed globular protein with the characteristics of a PIN-domain protein, such as an $\alpha/\beta/\alpha$ sandwich topology and four conserved acidic residues. The conserved residues are D8, E42, D97, and D115. The sandwich consists of five beta strands flanked by four N-terminal alpha helices and three C-terminal alpha helices. Despite low sequence identity, the PIN-domain fold is structurally conserved well. Four conserved acidic residues estimated to be catalytic residues are shown to cluster spatially together. (Figure 7.)

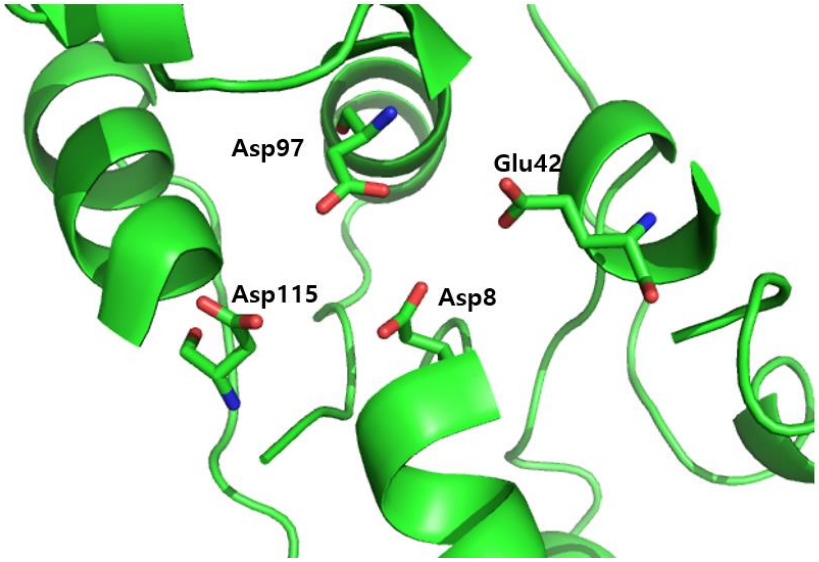

Figure7. Four conserved acidic residues

3.4 Homology search for Rv2757c

After determination of the Rv2757c toxin's crystal structure, DALI program was used to search structural homologues of Rv2757c. As a result, the Rv2757c toxin has high homology to several VapC proteins. Those VapC proteins that have a similarity to the Rv2757c toxin are shown in Figure 8. Among the homologues, the Rv0301 toxin was the most similar VapC homologue to the Rv2757c toxin and it has highly putative catalytic residues corresponding to D8, E42, D97, and D115 of the Rv2757c toxin by using sequence alignment function of Uniprot (http://www.uniprot.org). (Figure 9.) The superposition of the Rv2757c

toxin and the Rv0301 toxin with four conserved acidic residues labeled by sticks is shown in Figure 10.

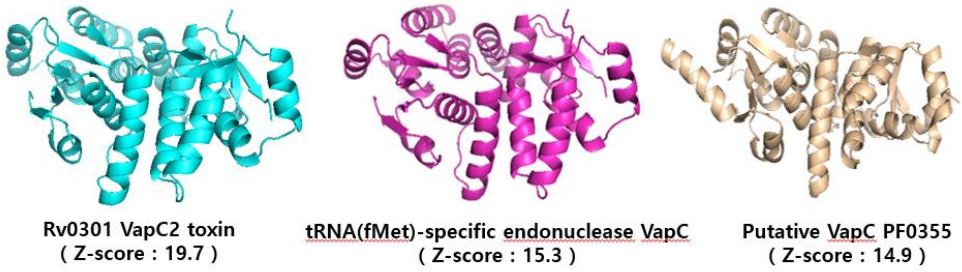

Figure8. The results of homology search for Rv2757c

```
1    -MTTRYLLDKSAAYRAHLPAVRHRLEPLMERGLLARCGITDLEFGVSARSREDHRTLGTY   59  O33299   VPC21_MYCTU
1    MTDQRWLIDKSALVRLTDSPDMEIWSNRIERGLVHITGVTRLEVGFSAECGEIARR--EF   58  O07228   VAPC2_MYCTU
         *:*:.***  *       .  .  :****:   *:* *.*.**.. *  *    :

60   RRD-----ALEYVNTPDTVWVRAWEIQEALTDKGFHRSVKIPDLIIAAVAEHHGIPVMHY  114  O33299   VPC21_MYCTU
59   REPPLSAMPVEYL--TPRIEDRALEVQTLLADRGHHRGPSIPDLLIAATAELSGLTVLHV  116  O07228   VAPC2_MYCTU
     *.       :**:     :  ** *:*  *:*:*.**. .***:***.**  *: *:*

115  DQDFERIAAITRQPVEWVVAPGTA-  138  O33299   VPC21_MYCTU
117  DKDFDAIAALTGQKTERLTHRPPSA  141  O07228   VAPC2_MYCTU
     *:**: ***:* * .* :.    :
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Figure 9. Sequence alignment of Rv2757c (VPC21) and Rv0301 (VAPC2)

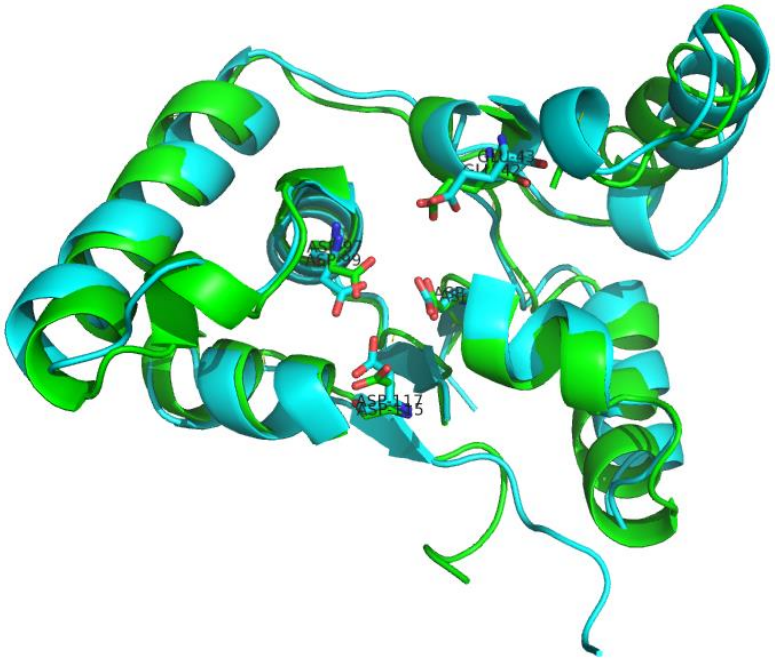

Figure 10. Superposition of Rv2757c and Rv0301

3.5 Ribonuclease activity assay of Rv2757c

Firstly, 4 μM of synthesized mRNA substrate coding the Rv2757c toxin was incubated in reaction buffer (200 mM NaCl, 20 mM sodium phosphate, 5 mM EDTA, pH 7.5) with 0, 0.25, 0.5, 2, 4 μM of Rv2757c for one hour at 37℃. mRNA coding Rv2757c for one hour at 37℃. 2.5% agarose gel shows from left to

right mRNA substrate with increasing concentration of the Rv2757C toxin. (Figure 11.)

Secondly, in the same condition except for pH and salt of buffer, 4 μM of RNA substrate was incubated with 2 μM of the Rv2757c toxin. As a result, mRNA substrate was cleaved in the range of buffer pH 5-7.5. (Figure 12.)

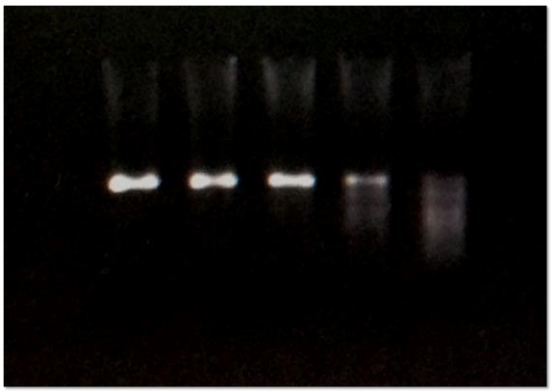

Figure 11. mRNA cleavage by increasing Rv2757c

Figure 12. mRNA cleavage in pH 5-7.5

Thirdly, 2 μM of purified mutants (D8A, E42A, D97A, D115A) were used in ribonuclease activity assay. 4 μM of mRNA substrate was cleaved in the reaction buffer by D8A and D115A as wild type, more cleaved by E42A than wild type, and there was no mRNA cleavage by D97A. (Figure 13.)

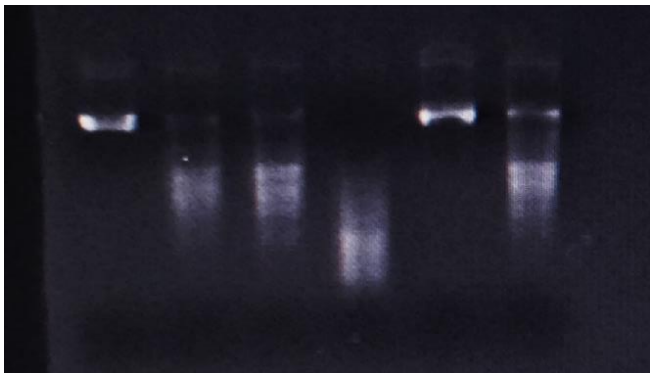

Figure 13. mRNA cleavage by Rv2757c and mutants (D8A, E42A, D97A, D115A)

Lastly, two fluorescence spectroscopy assay was performed. First assay was performed with fluorescent-labeled RNA substrate and 2 μM of the Rv2757c toxin in the reaction buffer adding 5 μM of several metal ions (Ca^{2+}, Co^{2+}, Mg^{2+}, Mn^{2+}, Ni^{2+}, Zn^{2+}) instead of EDTA for about 3 hours at 37℃. (Figure 14.) Fluorescence is measured when the substrate is cleaved which indicates the presence of ribonuclease activity. All ribonuclease activities with metal ion were

weaker than EDTA. In addition, ribonuclease activity of the Rv2757c toxin with Mg^{2+} or Mn^{2+} was much stronger than other metal ions.

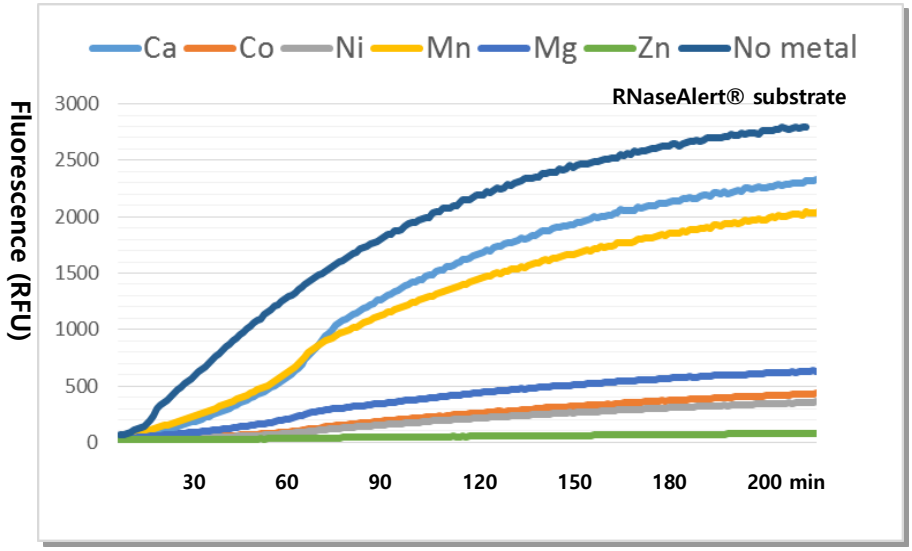

Figure 14. Fluorescence measurements of Rv2757c with different metal ions

Second spectroscopy was performed in the reaction buffer with 2 μM of the Rv2757c toxin and four mutants for 3 hours at 37℃. (Figure 15.) Compared to the result of gel electrophoresis, comparison of mutants was more clarified. D115A mutant also has weaker ribonuclease activity than wild type like D97A, and D8A also has stronger ribonuclease activity than wild type like E42A.

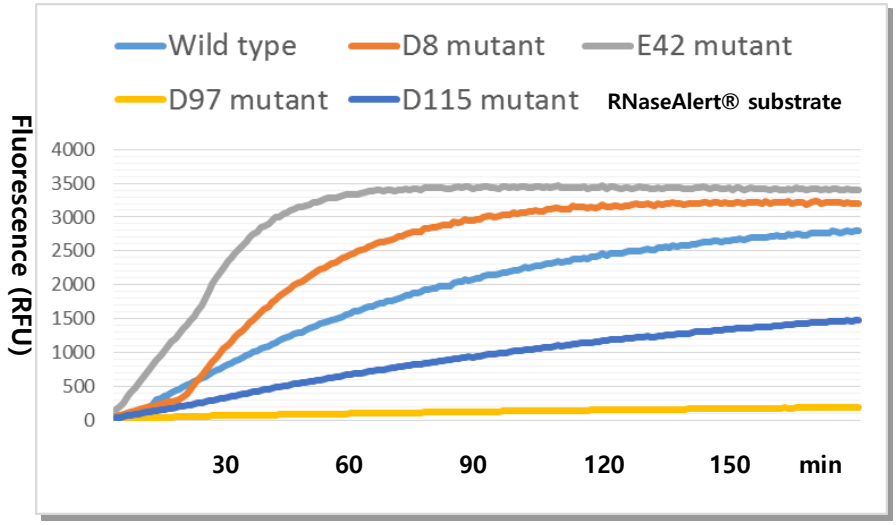

Figure 15. Fluorescence measurements of wild type and mutants

IV. DISCUSSION

The main objective of this study was the structural determination of the Rv2757c toxin. In order to achieve this goal, protein over-expression and purification was performed. With a lot of trials, we established purification methods for the Rv2757c toxin. Using several kinds of screening buffer, we could optimize crystallization condition and collect the X-ray diffraction data.

Determined crystal structure of the Rv2757c toxin is a two-fold axis dimer which consists of 138 amino acids for each subunit. It has seven α-helices, five β-strands and one 3_{10}-helix, showing the characteristics of a PIN-domain.

After determination of crystal structure, DALI program has been used to search structural homologues. Among several VapC proteins, the Rv0301 toxin was the most similar homologue to the Rv2757c toxin. According to sequence alignment, the Rv2757c toxin and the Rv0301 toxin has four conserved acidic residues thought to be catalytic-active residues in PIN domain. To determine whether these putative residues have actual roles in RNA degradation, ribonuclease activity assay was performed with wild type and four mutants of the Rv2757c toxin. After ribonuclease activity assay, two results were drawn.

Firstly, D8A and E42A have more increased activity than wild type, and D97A and D115A have more decreased activity than wild type. This means that

D97 and D115 could be the actual catalytic residues in the Rv2757c toxin, because D97A and D115 lost their most or part of ribonuclease activity.

Secondly, ribonuclease activity of the Rv2757c toxin decreases in the presence of divalent metals, in contrast to typical ribonucleases. Also, there are no metal molecules coordinating acidic residues in the crystal structure of the Rv2757c toxin. These results mean that there might be a unique mechanism of ribonuclease reaction in the Rv2757c toxin. Fortunately, the Rv2757c toxin has partial ribonuclease activity in the presence of Mg^{2+} or Mn^{2+} like as other ribonucleases do. Specifically, there is an ongoing debate if the catalytic activity of VapC toxins depend on one or two metal ions

The Rv2757c toxin was thoroughly characterized by several experiments as stated above. If the Rv2757c toxin is characterized by the crystal structure coordinating RNA substrate or the Rv2758c antitoxin, inhibition of the Rv2757c toxin in ribonuclease activity by steric hindrance for potential RNA targets can be clarified, and also these results can be an important part of the structural database of Toxin-Antitoxin system.

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국문초록

결핵균 (*Mycobacterium tuberculosis*)은 1882 년 Robert Koch 가 처음 발견하였다. 이는 감염성 균주로서 주로 호흡기를 통한 감염으로 폐질환을 야기한다. 결핵균은 세포표면에 waxy-coating 되어 있어 그람 염색법으로는 염색이 되지 않는다. 그람 염색법 대신 acid-fast 그람 양성균으로 분류된다. 결핵 환자들은 주로 약으로 치료하며 쓰이는 약물로는 isoniazid, rifampin, pyrazinamide, streptomycin 등이 쓰인다. 그러나 현재 전체 결핵 환자 중 25%정도가 항생제 내성 결핵균에 의한 것으로 파악되고 있다. 따라서 기존 결핵균 치료제와 다른 새로운 항생제 개발이 필요하다.

결핵균에는 78 개의 Toxin-Antitoxin 쌍이 존재한다. 박테리아가 영양부족, 항생제 처리 등 생존에 불리한 상황에 놓였을 때 toxin 은 세포의 성장을 저해하거나 세포사멸로 이끄는 역할을 한다. antitoxin 은 평상시에 toxin 과 결합하여 toxin 의 기능을 저해하고 있지만 위와 같은 상황에서 antitoxin 이 protease 에 의해 분해되어 toxin 에 대한 저해 효과가 사라진다. toxin 이 자유로워지면 박테리아의 대사와 성장이 저해되어서 항생제에 대한 감수성이 떨어지기 때문에, toxin 이 항생제 내성과도 관련이 있는 것으로 추정된다.

결핵균에 있는 Toxin-Antitoxin system 은 대부분 toxin 과 antitoxin 모두가 단백질로서 그 기능을 나타내는 type 2 Toxin-Antitoxin system 이다. 이중 Rv2757c 는 VapBC 계열에 속하는 VapC toxin 이다. 이 단백질의 3 차 구조를 구하기 이하여 원하는 gene 을 유전자 재조합 과정을 통하여 얻었으며, 이를 대장균 (E.coli, C41)에 형질전환하여 단백질을 발현시켰다.

Immobilized Metal Affinity Chromatography 를 이용하여 Hexa-histidine tag 가 붙은 Rv2757c 단백질을 정제하였고, Size-exclusion chromatography 과정을 거쳐 soluble 하고 순도 높은 Rv2757c 단백질을 얻을 수 있었다.

이 단백질을 Crystallization screen kit 를 이용하여 여러 조건의 용액에서 Rv2757c 의 crystal 을 얻을 수 있었다. 이 중 20% (w/v) PEG 8000, 100mM CHES/Sodium hydroxide pH 9.5 조건에서 생긴 crystal 로 X-ray diffraction data(100K, 1.5Å, Space group C2, unit cell parameters of a=61.465, b=44.813, c=57.486Å, α=γ=90, β=99.87°)를 얻을 수 있었다. 이를 PDB ID 3H87 의 toxin 단백질과 molecular replacement 를 진행하여 3 차 구조를 얻을 수 있었다.

Rv2757c 는 VapC toxin 으로서 RNase 활성을 가지고 있을 것으로 추정되기 때문에 in vitro Rnase test 도 진행하였다.

Key words: *Mycobacterium tuberculosis*, Toxin-Antitoxin System, Toxin, VapBC, Rv2757c, X-ray crystallography, SBDD(Structure-Based Drug Discovery)

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