國立臺灣大學生命科學院生化科學研究所

碩士論文

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Master Thesis

# 臺灣本土嗜熱菌 *Meiothermus taiwanensis* Lon 蛋白酵素功能與結構之研究

# Function-Structural Studies on the Lon Protease from *Meiothermus taiwanensis* WR-220

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# 國立臺灣大學碩士學位論文
# 口試委員會審定書

臺灣本土嗜熱菌 *Meiothermus taiwanensis* Lon

蛋白酵素功能與結構之研究

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*Meiothermus taiwanensis* WR-220

本論文係黃岱柏君（R97B46021）在國立臺灣大學生化科學研究所完成之碩士學位論文，於民國 99 年 6 月 29 日承下列考試委員審查通過及口試及格，特此證明。

口試委員：

（簽名）

（召集人）

(指導教授)

# 謝　誌

踏入生化科學領域，是直到我大學即將畢業時才突然湧現的想法，對於高中讀二類組且大學主修化學的我來說，生化領域是如此新奇且神秘。就讀碩士班的期間，能夠在中研院-如此優良的環境學習，我感到相當開心，因為這裡充滿了對科學的熱情，以及求知的慾望。學習的過程中，難免有些挫折，最後能夠完成這篇論文且順利完成學業，首先要感謝我的指導教授吳世雄老師，在學期間，老師不斷地勉勵我，實驗若遇到困難，也是不遺餘力地提供意見。感謝口試委員張文章老師、梁博煌老師、李岳倫老師，在口試時提供專業的指導，以及許多寶貴的意見。

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# 中文摘要

Lon 蛋白酵素高度保留存在於各種生物體中，且為多功能的單一聚合型酵素。先前的研究指出 Lon 蛋白酵素具有 ATPase、Protease、Peptidase、Chaperone、DNA-binding 等生物活性，透過其多功能的特性，Lon 蛋白酵素 可以維持生物體內蛋白質完整的功能與結構，或適時降解目標蛋白，進而調控體內蛋白質質量的恆定。本論文選擇臺灣本土烏來地區所分離出的嗜熱菌 *Meiothermus taiwanensis* (WR-220)，選殖出其基因體內被推論為 Lon 蛋白酵素的三個基因，透過表現與純化，以此三個蛋白酵素 (Mt-LonA1、Mt-LonA2、Mt-TTC1975) 為研究對象，探討此三個蛋白酵素在結構與功能的特徵，進而比較此三個蛋白酵素的差異。

Lon 蛋白酵素分為 A-type 與 B-type，其中 A-type Lon 具有三個功能區 (domain): N 端功能區、ATP 水解酵素功能區以及 C 端蛋白水解酵素功能區。透過應用軟體分析此三個蛋白酵素的一級結構，Mt-LonA1 與 Mt-LonA2 高度保留 A-type Lon 的三個功能區，而 Mt-TTC1975，只保留 C 端蛋白水解酵素功能區，因此我們認為 Mt-TTC1975 不應歸類為 Lon 蛋白酵素。

在結構方面，利用原二色偏光儀分析 Mt-LonA1、Mt-LonA2 與 Mt-TTC1975，結果顯示此三個蛋白酵素皆以 α-螺旋為主要的二級結構，並具有完整的三級結構。進一步利用分析級超高速離心儀、原態膠體電泳以及電子顯微鏡分析此三個蛋白酵素的四級結構，我們認為 Mt-LonA1 以六聚合體 (hexamer) 為主，Mt-LonA2 以二聚合體 (dimer) 與五聚合體 (pentamer) 為主，而 Mt-TTC1975 以六聚合體 (hexamer) 與七聚合體 (heptamer) 為主。

活性方面，Mt-LonA1 具有 ATPase、Protease、Peptidase、Chaperone 等活性，而 Mt-LonA2 具有 ATPase、Protease、Peptidase、DNA-binding 等活性，實驗結果顯示此兩個蛋白酶為 A-type Lon 蛋白酵素，值得注意的是，Mt-LonA1 缺乏 DNA-binding 的活性，而 Mt-LonA2 缺乏 chaperone 的活性，我們認為 Mt-LonA1

與 Mt-LonA2 在生物體內所扮演的角色可能不盡相同。而 Mt-TTC1975 則只擁有 chaperone 活性，此結果也與 Mt-TTC1975 不應歸類為 Lon 蛋白酵素的推論相符。

最後，我們討論 Mt-LonA1 與 Mt-LonA2 在 DNA-binding 活性的差異，藉由分析此兩個蛋白酵素與已被清楚研究的 Bt-Lon 之 α-domain 一級結構，以及比較 α-domain 的分子模型，我們認為 Mt-LonA2 之 K527 胺基酸與一段的保留序列 K-K-R ，可能為提供與 DNA 結合所需之正電荷的重要胺基酸。

中文關鍵詞：

Lon 蛋白酵素，嗜熱菌，四級結構

# Abstract

The Lon proteases had been known as one of the most evolutionarily conserved proteins. According to previous findings, Lon proteases possessed ATPase, protease, peptidase and chaperone activities. Based on these multi-functional Lon proteases, the protein quality control system and the regulation of metabolic process could both work well. In this study, the function-structural characterizations among Mt-LonA1, Mt-LonA2 and Mt-TTC1975 from *Meiothermus taiwanensis* would be juxtaposed to express the contrast.

The Lon protease family could be divided into two subfamilies, LonA and LonB, mainly based on the sources and the domain structures of these proteins. The LonA consisted of a variable N-terminal domain (N domain), a central ATPase domain (A domain), and a C-terminal protease domain (P domain) on a single polypeptide. Depending on the analysis of primary structures of Mt-LonA1, Mt-LonA2 and Mt-TTC1975, This study considered that Mt-LonA1 and Mt-LonA2 both should be classified as the LonA subfamily, for Mt-LonA1 and Mt-LonA2 both possessed the classical LonA-type domains. For Mt-TTC1975, it only possessed a high similarity in protease domain with canonical LonA. Therefore, it should not be classified as the Lon protease.

Structural characteristic results by circular dichroism showed that Mt-LonA1,

Mt-LonA2 and Mt-TTC1975 possessed mostly α-helical secondary structures and they all possesed well-defined three-dimensional structures. For quaternary structures, the AUC data, Native-PAGE and EM graph revealed that Mt-LonA1 functions mainly as a hexamer; the AUC data and Native-PAGE revealed that Mt-LonA2 might function as a mixture of dimer and pentamer; Native-PAGE revealed that Mt-TTC1975 functions as a hexamer or a heptamer.

Functional characteristic results showed that Mt-LonA1 exhibited the ATPase, protease, peptidase and chaperone activity; Mt-LonA2 exhibited the ATPase, protease, peptidase and DNA-binding activity; Mt-TTC1975 exhibited the chaperone activity only.

Lastly, the comparison of primary structure of α-domains and the results of homology modeling suggested that the K527 residue and the K-K-R conserved region of Mt-LonA2 might critically influence the DNA-binding activity.

Keywords:

*Meiothermus taiwanensis* WR-220, Lon protease, α-domain, quaternary structure

## Abbreviation Table

$AAA^+$: ATPase associated with various ellular activities

ATP: adenosine 5'-triphosphate

AUC: analytical ultracentrifuge

BSA: bovine serum albumin

CD: circular dichroism

DTT: dithiothreitol

IPTG: isopropyl-β-thiogalactopyranoside

Bt-Lon: *Bevibacillus thermoruber* Lon

Ec-Lon: *E. coli* Lon

Mt-Lon: *Meiothermus taiwanensis* Lon protease

Mt-TTC1975: *Meiothermus taiwanensis* TTC1975 peptidase

Glt-AAF-MNA: Glutaryl-Ala-Ala-Phe-methoxynaphthylamide

Suc-FLF-MNA: Succinyl-Phe-Leu-Phe-methoxynaphthylamide

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# 1. Introduction

## 1-1. Thermophiles

There were different types of thermal environments on the Earth that are populated by micro-organisms, adapted to live under high temperatures. Many of these environments were associated with volcanic activities. Hot springs, geysers volcanoes and deep-sea hydrothermal vents had all been popular hunting grounds for the search of new types of thermophilic micro-organisms. The existence of truly thermophilic bacteria was firmly established during Tom Brock's pioneering studies of the hot springs of Yellowstone National Park in the 1960s (*1*). However, in the last two decades, literatures had shown a surge of the academic interest in the world of micro-organisms living in extreme conditions. More and more extreme environments were explored because of technical limitation breakthroughs, and this had led to the exploration of the previously isolated resources of new organisms. These studies not only showed us how life functioning at environmental extremes, but also taught us much about the nature of lives and the possible properties of the first organisms that colonized the Earth when condition was far more rigorous than now. Extending from precedent researches, the relevant question of whether the existing extremophiles were only recently adapted to the unusual environments where they live, or they might be vestiges of ancient types of

organisms that evolved under extreme conditions on the early Earth.

Hence, a dramatic proliferation of research concerned with the thermophiles was seen in recent years. The major purpose of these researches including isolation, genome analysis, evolution and thermostable enzymes (*2*). There were abundant of studies oriented around *Thermus* genus and *Meiothermus* genus in Taiwan already. The major difference of *Thermus* genus and *Meiothermus* genus was their ideal growth temperature. *Thermus* genus could live at 70 $^{o}$C , and *Meiothermus* genus prefered to live at 55-66 $^{o}$C. Besides, the composition of polar lipid and fatty acid from cell membrane of *Thermus* genus and *Meiothermus* genus possessed significant difference. According to previous findings, two strains of a novel species, isolated from Wu-rai hot springs in the northern part of Taiwan were found. These isolates were aerobic, thermophilic, non-sporulatng, red-pigmented and heterotrophic. Also, they formed extremely long, filamentous trichomes from cells of different lengths. The name *Meiothermus taiwanensis* sp. nov., was proposed for this novel species. One isolate, designated WR-220, was chosen for this study (*3*).

**1-2. Lon protease**

**Background overview**

The Lon protease had been known as one of the most evolutionarily conserved

proteins. Its homologues presented in archaea, prokaryotes (*4-6*), and certain cellular compartments (mitochondria, peroxisomes, and plastids) in eukaryotes (*4-11*). Lon protease was also known as CapR or La protease, and it was the first ATP-dependent protease that was discovered from *Escherichia coli* (*12-13*). Its name was derived from the long undivided filaments phenotype of *E. coli lon*$^-$ mutant, either upon UV irradiation or when grown in nutrient broth without aeration (*14*).

Lon protease (EC 3.4.21.53) had been classified as a member of the AAA$^+$ (ATPases associated with diverse cellular activities) super-family. This super-family was characterized by a conserved segment of 220-250 amino acids, referred to as an α-domain or nucleotide binding domain, which contained several conserved motifs for ATP binding and hydrolysis (*15*). This super-family included Lon and other known members; for example, FtsH, ClpAP, ClpXP, ClpC, and HslVU were all ATP-dependent proteases (*16*). Lon and FtsH protease carried both the ATPase and the proteolytic active sites within a single polypeptide chain. In the Clp family, these functions were encoded by separate polypeptide chains (*17*).

Currently, the Lon protease family could be divided into two subfamilies, LonA and LonB (Fig. 1-2), mainly based on the sources and the domain structures of these proteins (*18*). The LonA consisted of a variable N-terminal domain (N domain), a central ATPase domain (A domain), and a C-terminal protease domain (P domain) on a

single polypeptide. LonB consisted only the later two domains, A and P, but had a membrane-spanning domain between the Walker motif A and B within the ATPase domain that anchors the protein to the cytoplasmic side of the membrane (*19*). Most Lon studies had been focusing on LonA while relatively little researches were conducted on LonB. The complete genomic data of bacterial and archaeal species revealed that a significant number of Lon proteases were identified as belonging to the LonB subfamily (*20*).

**Functions**

It had been known for a long time that serine residue played an important role at the P domain active site of Lon protease (*21*). Recently, Birghan identified a region in virus protein 4 (VP4) related to the protease domain of Lon proteases that used Ser-Lys dyad as a catalytic module (*22*). The significant similarity of amino acid sequences of proteases Lon and repressors LexA in the regions, including the catalytic serine and lysine residues, revealed different families of the enzymes that might utilize similar Ser-Lys catalytic dyad (*23*). Furthermore, the crystal structure also indicated that Lon protease had a Ser-Lys dyad at the P domain active site(*24-27*). However, Lon was not only an ATP-dependent protease, but also a chaperone, or even a DNA-binding protein (*31-32*). For example, *in vitro* experiments indicated that DNA binding stimulated the

ATP-dependent proteolysis and protein-dependent ATPase activity of Lon (*28*), and the DNA binding affinity could be regulated by protein substrates (*34-35*). On the other hand, this study knew protein quality control played the key roles in prokaryotes and eukaryotes by depicting cellular surveillance systems of structural and functional integrity of proteins inside a cell (*29-30*), and the major components of the protein quality control system were molecular chaperones and proteases. Molecular chaperones helped to promote proper protein folding and prevent aggregation, and energy-dependent proteases irreversibly helped to degrade the damaged or denatured proteins which cannot be rescued by chaperones (*31*). Oligomeric ATP-dependent proteases were consisted of the chaperone, and the protease machinery carry out the protein quality control in prokaryotes and eukaryotes. In eukaryotic cells, the multimeric 26S proteasomes had degraded target proteins covalent covalently modified with ubiquitin. In bacterial cells, a number of ATP-dependent protease often functioning both as chaperone as well as proteases in bacterial cells; such as, Lon, FtsH, ClpAP, ClpXP, and HslVU(*29*).

The α-domain was a small sub-domain located in the ATPase domain and typically contained four of five α-helices (*32-33*). This domain could only be recognized in all defined $AAA^+$ proteins; therefore, the α-domain had been used to characterize the $AAA^+$ super-family. According to previous findings, the α-domain, expressed from Lon and

several Clp family members independently, could recognize their specific protease substrated. Hence, the α-domain had also been called the Substrate Sensor and Discriminatory (SSD) domain (*34*). This study would refer to this domain as the α-domain because other substrated binding domains were found among the $AAA^+$ proteins (*35*).

**Quaternery structure**

Lon functions as a homo-oligomer (*43-46*), and which was distinctive from the other ATP-dependent proteases that formed hetero-oligomer like Clp/HSP100 proteins. Nevertheless, the oligomeric state of Lon protease still remained blur. Lon behaved as a hexamer in *E. coli (36)*, as a tetramer to a hexmer in *Mycobacterium* (*37-38*), and as a hexamer or a heptamer in yeast mitochondria (*37-38*).

**Three putative Lon proteases of *Meiothermus taiwanensis***

In the genome of *Meiothermus taiwanensis* WR-220, three genes were annotated as ATP-dependent protease Lon. Sequence comparisons indicated that two of genes showed apparent similarities with bacterial LonA-type proteases; such as, Ec-Lon protease, especially from regions corresponding to domain of ATP-binding and hydrolysis and domain of proteolysis. In order to distinguish these two gene products,

this study named them Mt-LonA1 and Mt-LonA2. The third gene product had been named TTC1975 peptidase (*39*), which exhibited a similarity only at C-terminal proteolytic domain. In this study, the function-structural characteristic among Mt-LonA1, Mt-LonA2 and Mt-TTC1975 would be juxtaposed to show contrast.

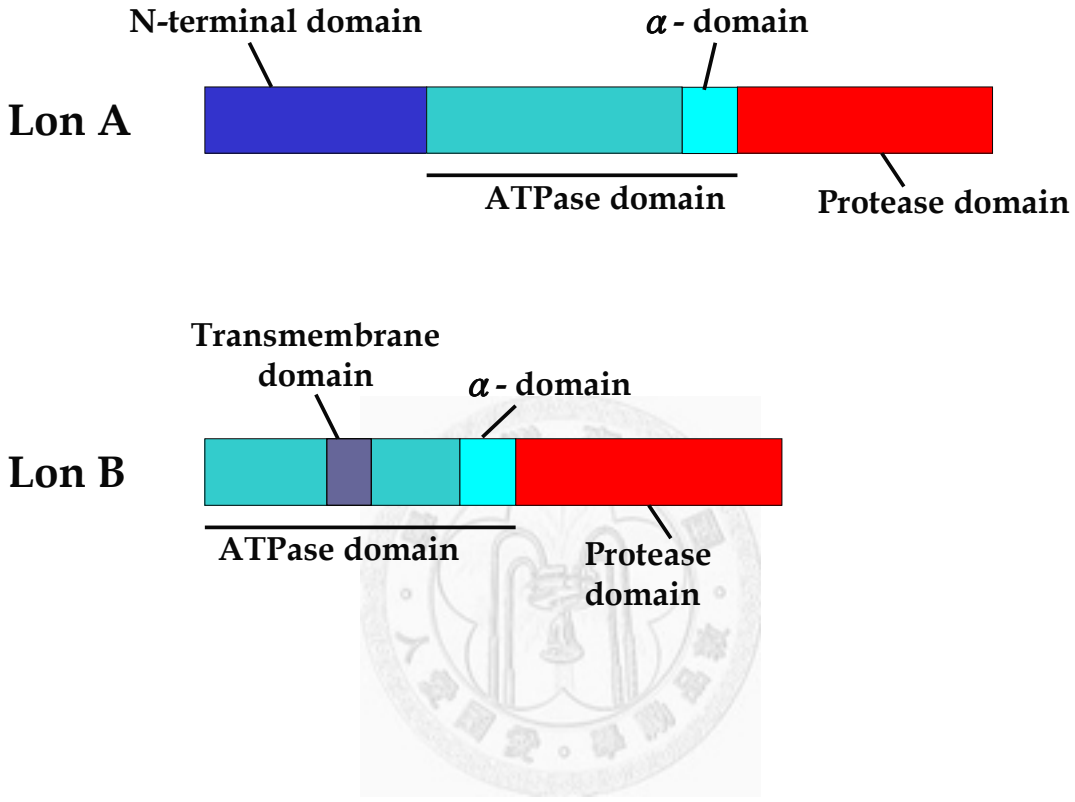


**Figure 1-1. Domain structures of LonA and LonB subfamily**

The Lon protease family could be divided into two subfamilies, LonA and LonB, which the division mainly based on the sources and the domain structures of these proteins.

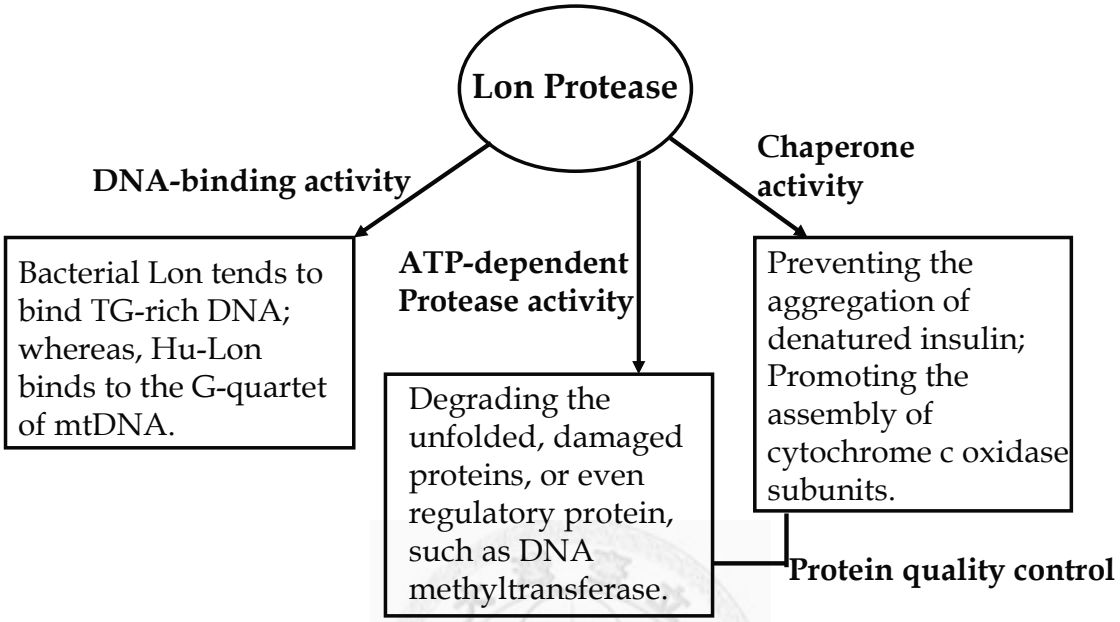


**Figure 1-2. Schematic diagram of the functions of Lon**

Lon had been known as a multi-function protein. The protease activity and chaperon activity were considered to be very influential in protein quality control. (Nucleic Acids Res. 2008 Mar;36(4):1273-87. Epub 2008 Jan 3)

# 2. Materials and Methods

## 2-1. Materials

### Equipments

1. Circular dichroism JASCO J-715 spectropolarimeter (Jasco, Japan)

2. Ultraspec 4000 UV/Visible spectrophotometer (Pharmacia Biotech)

3. Beckman XL-A analytical ultracentrifuge (Beckman Instruments, Fullerton, CA)

4. Electron microscope (JEOL 1400)

5. CCD (Gatan UltraScan)

6. UV/Vis spectrophotometer (DU-730, Beckman Coulter)

7. Jobin Yvon Fluorolog-3 fluorescence spectrophotometer (Edison, NJ)

8. Spectrophotometer (Ultraspec 4000 UV/Visible, Pharmacia Biotech)

### Enzymes, Bacterial strains, Plasmid DNA

1. PfuUltra HF DNA polymerase (Stratagene, USA)

2. T4 DNA Ligase (Fermentas, CANADA)

3. Restriction enzyme NdeI (Fermentas, CANADA)

4. Restriction enzyme HindIII (Fermentas, CANADA)

5. *Meiothermus taiwanensis* WR-220

6. *E.coli* starin JM109

7. *E. coli* strain BL21 (DE3) (stratagene)

8. Plasmid pGEM-7zf(+) (Promega, USA)

9. Plasmid pET21a(+) (Novagen, Merck, Germany)

**Chemicals, Buffers, Peptides**

1. LB Broth (Bioshop, CANADA)

2. Ampicillin (Bioshop, CANADA)

3. IPTG: isopropyl-β-thiogalactopyranoside (Bioshop, CANADA)

4. Lysis buffer (50 mM Tris-HCl pH 8.0, 300 mM NaCl, 10 mM imidazole, 20% glycerol)

5. Ni Sepharose 6 Fast Flow resin (GE healthcare, USA)

6. Econo-Pac column (BioRad, USA)

7. Washing buffer (50 mM Tris-HCl pH 8.0, 300 mM NaCl, 40 mM imidazole)

8. Elution buffer (50 mM Tris-HCl pH 8.0, 300 mM NaCl, 400 mM imidazole)

9. Amicon Ultra-15 (Millipore, USA)

10. Dialysis buffer: PBS (pH 7.4) containing 10% glycerol and 5 mM $MgCl_2$

11. CD buffer (10 mM $Na_2HPO_4$, 2 mM $KH_2PO_4$, 3 mM KF and 70 mM NaF, pH 7.4)

12. 10% native gel (CosmoPAGE, Nacalai, USA)

13. CosmoPAGE Native run buffer (0.8 M Tricine, 1.2 M Tris, 1 mM Sodium Bisulfite, pH 8.3)

14. CosmoPAGE Native Sample buffer (40% Glycerol, 0.38 M Tris, 0.3 M Acetic Acid, 4% Ficoll-400, 0.02% Phenol Red, 0.02% Brilliant Blue G250, 8 mM EDTA-2Na, pH 7.7, Nacalai USA)

15. MG/AM solution [0.045% malachite green hydrochloride (MG) : 4.2% ammonium molybdate in 4N HCl (AM) = 3:1 ]

16. FITC-α-casein (type I, Sigma-aldrich)

17. CHES-Na (pH 12.0, Sigma-aldrich)

18. Fluorogenic peptides containing 4MNA (4-methoxy-β-naphtjyl-amide): Suc-FLF-MNA and Glt-AAF-MNA

19. Bovine insulin

20. EMSA reaction buffer: 50 mM Tris-HCl (pH 8.0), 10 mM $MgCl_2$, 150 mM NaCl

## 2-2. Methods

**Promoter prediction**

The putative promoter regions of Mt-LonA1 and Mt-LonA2 were predicted by the Website: Berkeley Drosophila Genome Project. (http://www.fruitfly.org/seq_tools/promoter.html)

**Sequence alignment**

Both full-length protein sequences and α-domain protein sequences were aligned by program DNAstar MegAlign (ClustalV Method).

**Bacterial strain and culture condition**

*Meiothermus taiwanensis* WR-220, with ideal growing temperature 55-60 $^{o}$C was cultivated at 55 $^{o}$C in a liquid *Thermus* broth medium (*40*).

**Cloning of Mt-lonA1 and Mt-lonA2**

Total genome of *M. taiwanensis* was sequenced and the DNA sequence related to Lon protease was annotated.

In order to study, the DNA fragments encoding Mt-LonA1 and Mt-LonA2, flanked by NdeI and HindIII sites, were amplified from the chromosomal DNA of *Meiothermus taiwanensis.* PfuUltra HF DNA polymerase (Stratagene, USA) was used, and the primers were listed in Table 6-1. The amplified fragments which with restriction enzyme cutting seats were ligated with plasmid pGEM-7zf(+) (Promega, USA); recombinant plasmids were introduced into *E.coli* JM109 by transformation. Transformants were checked by blue-white selection and PCR. Plasmid DNA was

isolated from the transformants and digested within NdeI and HindIII; the fragments were ligated with plasmid pET21a(+) (Novagen, Merck, Germany), which encoding a 6×His tag. The DNA sequences of all constructed plasmids were checked via sequencing.

**Expression and purification of Mt-LonA1 and Mt-LonA2**

The clones of Mt-LonA1 and Mt-LonA2 were over-expressed in *E. coli* strain BL21 (DE3) (stratagene), and the His-tagged proteins were purified as the following. Both overnight bacterial cultures were inoculated into 1 L LB medium (containing 100 μg/mL ampicillin) and later grown to 0.6 in $OD_{600}$ at 37 °C. Expressions of Mt-LonA1 and Mt-LonA2 were then induced for 4 hours with 1mM IPTG at 37 °C.

The bacterial cultures were centrifuged at 9,000×g for 30 minutes, and the pellet was re-suspended in lysis buffer (50 mM Tris-HCl pH 8.0, 300 mM NaCl, 10 mM imidazole, 20% glycerol) and disrupted by ultrasonication. The cell debris was removed by centrifugation at 40000×g for 30 minutes at 4 °C. The supernatant was applied to Ni Sepharose 6 Fast Flow resin (GE healthcare, USA) and gently mixed for 30 minute at roomtemperature. The resin mixture was packed into an Econo-Pac column (BioRad, USA) and washed with washing buffer (50 mM Tris-HCl pH 8.0, 300 mM NaCl, 40 mM imidazole), and the nonspecific binding molecules were subsequently flushed out

with the washing buffer. The protein was eluted with the elution buffer (50 mM Tris-HCl pH 8.0, 300 mM NaCl, 400 mM imidazole). Purified protein was collected and concentrated by using Amicon Ultra-15 (Millipore, USA), and later, it was dialyzed against PBS buffer (pH 7.4) containing 10% glycerol and 5 mM $MgCl_2$.

The protein concentration was determined by measuring the absorbance of sample at 280 nm (*41*). The purity of protein was examined by SDS-PAGE and coomassie brilliant blue staining.

**Circular Dichroism spectroscopy**

CD spectroscopy was an ideal technique for speedy estimation of the secondary structure composition of proteins (*42*). The differences in absorption between left and right circular polarized light were defined. CD intensity was often given in terms of ellipticity(θ), which represented the ratio of the major and minor axes of the elliptically polarized light emerging from a sample.

In this study, circular dichroism spectra were recorded on the JASCO J-715 spectropolarimeter (Jasco, Japan). Protein concentrations were 0.7 μM (far-UV region) and 20 μM (near-UV region) in CD buffer (10 mM $Na_2HPO_4$, 2 mM $KH_2PO_4$, 3 mM KF and 70 mM NaF, pH 7.4). The 0.1 cm light path cuvette was used for far-UV, and the 1 cm one was used for near-UV.

**Thermal denaturation assay**

Thermal denaturation assay was tested by using the Ultraspec 4000 UV/Visible spectrophotometer (Pharmacia Biotech). The protein solution (9.25 μM) were heated from 20 to 95 $^{o}$C at 1 $^{o}$C min$^{-1}$ , with a 1 cm light path cuvette, and the aggregation was monitored by measuring the light scattering at 360 nm.

**Analytical ultracentrifuge**

The quaternary structure of Mt-LonA1 and Mt-LonA2 was analyzed by a Beckman XL-A analytical ultracentrifuge (Beckman Instruments, Fullerton, CA) with an An60Ti rotor. Sedimentation velocity was performed at 20,000 rpm with standard double-sector centerpieces at 20 $^{o}$C. The UV absorption (280 nm) of the cells was scanned 250 times every 4 min. The data was analyzed with the SedFit program (*43*). All samples were diluted to 0.5 in $OD_{280}$ (10 μM) with PBS buffer (pH 7.4) containing 10% glycerol and 5 mM $MgCl_2$. All were visually checked for clarity after ultracentrifugation, and no precipitation was observed.

**Native polyacrylamide gel electrophoresis**

Native PAGE of proteins were performed on 10% native gel (CosmoPAGE,

Nacalai, USA), using CosmoPAGE Native run buffer (0.8 M Tricine, 1.2 M Tris, 1 mM Sodium Bisulfite, pH 8.3). Protein samples were prepared in CosmoPAGE Native Sample buffer (40% Glycerol, 0.38 M Tris, 0.3 M Acetic Acid, 4% Ficoll-400, 0.02% Phenol Red, 0.02% Brilliant Blue G250, 8 mM EDTA-2Na, pH 7.7, Nacalai USA). The gels were ran in 200 V for 5-7 hours and stained by either coomassie brilliant blue or SYPRO-Ruby.

**Transmission electron microscopy**

Samples of Mt-Lon [ ~0.08 mg/ml, in 12 mM NaCl, 0.8 mM $NaH_2PO_4$ (pH 7.0)] were applied to a carbon-coated grid and stained by Uranyl acetate. EM imaging was performed under low-dose conditions on a 120 kV electron microscope (JEOL 1400, 120,000 magnified), and the images were recorded on a CCD (Gatan UltraScan: 15 mm, 4K by 4K).

**ATPase assay**

The basal ATPase activity assay was performed for inorganic phosphate as described previously (*44*). This assay was based on quantification of the green complex formed between malachite green, molybdate and free orthophosphate. The rapid color formation from the reaction could be conveniently measured on a spectrophotometer

(600-660 nm). Briefly, reaction mixture was composed of 50 mM Tris-HCl (pH 8.0), 10 mM $MgCl_2$, 1 mM ATP, and 4 μg of Lon protease in a total volume of 100 μL. Reaction mixtures were incubated at 55 °C for 30 minutes. The color of reaction was developed by adding 800 μL MG/AM solution [0.045% malachite green hydrochloride (MG) : 4.2% ammonium molybdate in 4N HCl (AM) = 3:1 ]. The development of color was stopped by adding 100 μL 34% sodium citrate. The optical density of the reaction was determined at 660 nm in a UV/Vis spectrophotometer (DU-730, Beckman Coulter).

**Protease assay**

The protease activity assay was performed as described previously (*45*). Reaction mixture was composed of 50 mM Tris-HCl (pH 8.0), 10 mM $MgCl_2$, 1 mM ATP, 10 μg FITC-α-casein (type I, Sigma-aldrich) and 4 μg of Lon protease in a total volume of 200 μL. Reaction mixtures were incubated at 55 °C for 60 minutes. 10 μL of 10 mg/mL BSA and 100 μL of 10% trichloroacetic acid (TCA) were added and kept on ice for 10 minutes. The mixture was centrifuged at 13,000 rpm for 10 minutes (to remove pellet). The supernatant was mixed with 200 μL of 0.5 M CHES-Na (pH 12.0, Sigma-aldrich). Fluorescence was measured in a Jobin Yvon Fluorolog-3 fluorescence spectrophotometer (Edison, NJ) with an excitation wavelength of 490 nm and an emission wavelength of 525 nm.

**Peptidase assay**

The peptidase activity assay was performed as described previously (*46*). The reaction mixture was composed of 50 mM Tris-HCl (pH 8.0), 10 mM $MgCl_2$, 1 mM ATP, 0.3 mM fluorogenic peptide, and 4 μg of Lon protease in a total volume of 200 μL. Reaction mixtures were incubated at 55 °C for 60 minutes and stopped by the addiction of 100 μL of 1% SDS and 1.2 mL of 0.1 M sodium borate (pH9.2). Fluorescence was measured in a Jobin Yvon Fluorolog-3 fluorescence spectrophotometer (Edison, NJ) with an excitation wavelength of 335 nm and an emission wavelength of 410 nm. Fluorogenic peptides containing 4MNA (4-methoxy-β-naphtjyl-amide), Suc-FLF-MNA or Glt-AAF-MNA were used in this experiment.

**Chaperone activity assay**

The assay was based on preventing the aggregation of denatured insulin (*47*). Bovine insulin (52.4 μM) in PBS buffer at pH 7.4 was unfolded by adding DTT to reach 20 mM as the final concentration at 37 °C. The aggregation of insulin was monitored by measuring the absorption due to light scattering at 360 nm in a spectrophotometer (Ultraspec 4000 UV/Visible, Pharmacia Biotech) for 30 minutes. The protein concentration was 13.1 μM for Mt-LonA1and Mt-LonA2; whereas, 52.4 μM was for

Mt-TTC1975.

**Electrophoretic mobility shift assay**

EMSA was also known as GMSA (Gel Mobility Assay), which was a simple method for detecting the interaction between protein and nucleic acid (*57-58*). This assay was fast and convenient for observing the electrophoretic mobility of a protein-nucleic acid complex. Briefly, mixtures of protein and nucleic acid were likely subjected to electrophoresis using agarose gel. The electrophoretic mobility of DNA would be changed when the protein is bound to the DNA.

In this experiment, the reaction mixture was composed of 50 mM Tris-HCl (pH 8.0), 10 mM $MgCl_2$, 150 mM NaCl, 2.5-10 μg of Lon protease, and 450 ng of plasmid DNA (pUC-19) in a total volume of 20 μL. Reaction mixtures were incubated at 55 $^{o}$C for 5 minutes and analyzed by gel electrophoresis in 1% agarose. DNA bands were then visualized by ethidium bromide staining.

**Homology modeling**

Three-dimensional, atomic-scale models of Mt-Lon α-domain were developed by exploiting the sequence similarity to the Bt-Lon and Ec-Lon α-domain. In short, MODELER (*48*) program encoded in InsightII was used to build up the model structure

with spatial restraint method. For multiple-alignment of Mt-Lon α-domain sequence with Bt-Lon and Ec-Lon α-domain, MODELER yielded 10 models, each of which contained three optimizing loop structures. The structure with lowest violation score and lowest energy was chosen as candidate. Several structural validation tools were adopted to check the model quality. The distribution of the backbone dihedral angles of the model was evaluated by the representation of Ramachandran plot using PROCHECK (*49*). The Prostat module of InsightII was used to analyze the properties of bonds, angles, and torsions. Profile three-dimensional program (*50*) was used to check the structure and sequence compatibility. The electrostatic potential of the modeled Mt-Lon α-domain was calculated by the program ABPS (*51*) and presented by pymol (http://www.pymol.org).

# 3. Result

## PART 1: Analysis of the three putative Lon proteases from *Meiothermus taiwanensis* WR-220

### Putative promoter region of Mt-LonA1 and Mt-LonA2

Using the tool as described in Materials and Methods, this study predicted the putative promoter regions of Mt-LonA1 and Mt-LonA2, and the transcription start site in the promoter region was marked respectively. Moreover, This study found an unusual repetition, the GATAGATA sequence, near the -10 region for both of Mt-LonA1 and Mt-LonA2. The GATA sequence was a consensus sequence that could be recognized and bound by GATA transcription factor family (*52*). Consequently, this GATA sequence might be crucial for the regulation for the genes of Mt-LonA1 and Mt-LonA2.

### Lon protease amino acid sequence alignment

This study aligned the amino acid sequences of the commonly studied Lon proteases with Mt-LonA1 and Mt-LonA2 (Fig. 5-2, 5-3, 5-4), trying to find the relationship between them, regarding the evolution. As a result, Mt-LonA1 showed 73.8% identity compared to *Thermus thermophilus* HB27 LonA1. It had been referred

to A1 because it is identical to Mt-LonA1. *Thermus thermophilus* HB27, a famous thermophile, that had been widely studied, yet the previous studies had not categorized its Lon in detail. When compared with Bt-Lon and Ec-Lon, Mt-LonA1 shared 48.1% and 49.6% identity, respectively (Fig. 5-2). As for Mt-LonA2, it had 77.5% identity compared to *Thermus thermophilus* HB27 LonA2.It had been referred to A2 because it is identical to Mt-LonA2. And when compared with Bt-Lon and Ec-Lon, Mt-LonA2 shared 47.6% and 44.6% identity, respectively (Fig. 5-3). Next, this study compared its own Mt-LonA1 and Mt-LonA2 and found 45.2% identity between them (Fig. 5-4), meaning that they might play different roles in *Meiothermus taiwanensis* WR-220. Lastly, this study came up with the graph showing phylogenetic tree that consisted of the commonly studied Lon proteases and three putative Lon proteases in *Meiothermus taiwanensis* WR-220 (Fig. 5-5). The graph revealed the LonA2 group of *thermus/meiothermus* genus delegated from LonA-type protease at early stage, then the remainings split into LonA1 group and commonly LonA-type group, representing that the characteristics of LonA1 group might be close to the commonly LonA-type group. And obviously, the TTC1975 peptidase group was distant from the groups mentioned above. Besides, this study found the conserved Ser-Lys dyad at the active site of proteolytic domain in all Lon proteases that this study chose to align. The putative primary structures of α-domain this study predicted by these alignments depending on

previous studies (*39, 64-65*)

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**PART 2: Structural characteristic and thermal stability of Mt-LonA1, Mt-LonA2 and Mt-TTC1975**

**Expression and purification**

This study cloned Mt-LonA1 and Mt-LonA2 into pET21a(+) vector and expressed the gene products by *E. coli.* BL21(DE3). The Mt-TTC1975 clone was constructed and expressed by Dr. Liao in this study's lab. After purification, Mt-LonA1 and Mt-LonA2 both showed a single band near 90,000 Da (Fig. 5-6). The molecular mass of Mt-LonA1 and Mt-LonA2 this study estimated were to be 89,368 Da and 92,057 Da, respectively. For Mt-TTC1975, the molecular mass was estimated 78,949 Da, and result showed a single band near 80,000 Da (Fig. 5-6).

**Secondary and tertiary structure**

Circular dichroism (CD) gave this study much useful biophysical information about the secondary structures of Mt-LonA1, Mt-LonA2 and TTC1975 peptidase. The characteristics of the far-UV CD spectra of Mt-LonA1, Mt-LonA2 and TTC1975 peptidase were very similar (Fig. 5-7). A negative band was at about 222 nm due to the

$n\pi^*$ transition, and a negative band was at about 209 nm due to the parallel and perpendicular components of $\pi\pi^*$ transition that were measured in milli-degrees by far-UV CD, suggesting that Mt-LonA1, Mt-LonA2 and Mt-TTC1975 possessed major α-helical secondary structures. The CD data were then analyzed by the popular programs: CONTIN, SELCON and CDSSTR (*53*), for the estimation and the detail of secondary structure (Table 6-2).

The near-UV CD spectra showed significant peaks at 260-300 nm reflecting the microenvironments of bulky tryptophan, tyrosine, phenylalanine residues of Mt-LonA1, Mt-LonA2 and Mt-TTC1975 indicating that they possessed well-defined three-dimensional structures (Fig. 5-8). The far-UV and near-UV CD spectra showed that Mt-LonA1, Mt-LonA2 and Mt-TTC1975 were folded into a well-defined structure. However, the Mt-TTC1975 exhibited a higher intensity of signals that differed from Mt-LonA1 and Mt-LonA2; a reasonable result for the primary structure identity. Mt-LonA1 had 45.2% identity compared to Mt-LonA2. And Mt-TTC1975 shared 12.8% and 14.7% identity respectively, when compared with Mt-LonA1 and Mt-LonA2.

**Thermal stability**

The thermal stability of Mt-LonA1, Mt-LonA2 and Mt-TTC1975 were analyzed by tracing aggregation of soluble protein (Fig. 5-9). Protein solutions were heated from 25

$^{o}$C to 95 $^{o}$C, and the turbidity was recorded due to the light scattering at 360 nm. As a result, the Mt-LonA1 became turbid at 66 $^{o}$C, and Mt-LonA2 and Mt-TTC1975 became turbid at 70 $^{o}$C, 74 $^{o}$C, respectively.

**Quaternary structure**

Previous studies had revealed that Lon protease had homo-oligomeric structure (*43, 45-46*). The native molecular mass of Mt-LonA1 and Mt-LonA2 were estimated by analytical ultracentrifuge (AUC). The sedimentation coefficients (S or Svedberg values) were determined with SEDFIT (*43*). The sedimentation coefficient distribution of Mt-LonA1 was between 8S and 26S with three major peaks at 9.8S, 13.3S and 17.7S (Fig. 5-10), and the molecular weights of these major peaks were estimated 342892, 537526 and 827224, respectively. Based on the 89368 molecular weight of Mt-LonA1, these major peaks of Mt-LonA1 might represent a 3.8 mer, a 6.0 mer and a 9.2 mer native quaternary structure, respectively. On the other hand, the sedimentation coefficient distribution of Mt-LonA2 was between 8S and 30S with two major peaks at 10.4S and 16.5S (Fig. 5-11), and the molecular weights of these two major peaks were estimated 222788 and 450227 respectively. Based on the 92057 molecular weight of Mt-LonA1, these two major peaks of Mt-LonA2 might represent a 2.4mer and a 4.9mer native quaternary structure, respectively.

To examine the quaternary structures of Mt- LonA1 and Mt-LonA2 that were estimated by AUC, this study chose another simple technique: Native-PAGE. Consequently, the Native-PAGE of Mt-LonA1 showed a significant band at about 550 kDa (Fig. 5-12A), proving that the hexamer quaternary structure of Mt-LonA1 was true, but this study did not find any other clear bands of Mt-LonA1 on Native-PAGE. Based on the Native-PAGE graph (Fig. 5-12A), this study did not see any bands of Mt-LonA2; therefore, this study set the same experiment but the gels, which were stained by SYPRO-Ruby for higher sensitivity (Fig. 5-12B). The result showed several bands of Mt-LonA2, two of them were about 450 kDa and 220 kDa. These two significant bands were same as the result that indicated by AUC, suggesting that Mt-LonA2 might have both dimmer quaternary structure and pentamer quaternary structure under native condition. Besides, some minor bands were below 140 kDa or even 66 kDa markers, and this study knew the molecular weight of Mt-LonA2 is about 90 kDa; therefore, the bands which were below the 66 kDa marker, might be the self-digested segments of Mt-LonA2. Lastly, the bands between the 140 kDa marker and 66 kDa marker might be the complex of monomer and self-digested segments of Mt-LonA2.

Moreover, this study want to obtain more evidences of the quanternary structures of Mt-LonA1 and Mt-LonA2; thus, the transmission electron microscopy was employed for this purpose. Negative staining of Mt-LonA1 with uranyl acetate showed some

regular particles (Fig. 5-13A). To magnify these particles, this study found a particle that clearly showed a ring-shaped homohexamer structure of Mt-LonA1 (Fig. 5-13B). On the other hand, this study did not find any significant particles of Mt-LonA2 unfortunately (data not shown).

**PART 3: Functional characteristics of Mt-LonA1, Mt-LonA2 and Mt-TTC1975**

**ATPase activity**

According to analysis of primary structures of Mt-LonA1, Mt-LonA2 and Mt-TTC1975, this study found that Mt-LonA1 and Mt-LonA2 both had a conserved ATPase domain, but Mt-TTC1975 did not have, suggesting that Mt-LonA1 and Mt-LonA2 might exhibit the ability of ATP hydrolysis, but Mt-TTC1975 did not. As a result (Fig. 5-14), Mt-LonA1 and Mt-LonA2 both showed significant ability of ATP hydrolysis. Mt-TTC1975 showed an unexpected, lower activity, which might be an experimental error.

**Protease activity**

Lon was an ATP-dependent protease; therefore, the protease domain must worked with the ATPase domain in the presence of ATP. As the primary structure analyzing

result, this study considered that Mt-LonA1 and Mt-LonA2 might exhibit the protease activity, but Mt-TTC1975 does not. As a result of protease activity (Fig. 5-15), Mt-LonA1 and Mt-LonA2 both showed the expected protease activity, and Mt-TTC1975 showed very tiny fluorescence intensity, so that Mt-TTC1975 does not exhibit the protease activity.

**Peptidase activity**

This study contemplates on whether Mt-LonA1, Mt-LonA2 and Mt-TTC1975 had the peptidase activity. For the purpose, this study used a simple method that was described in Materials and Methods chapter. As a result (Fig.5-16A, 5-16B), Mt-LonA1 and Mt-LonA2 clearly cleaved both fluorogenic peptide. For Mt-TTC1975, although this study saw the slight fluorescence signal, but it was still a very weak signal when compared to the signal of Mt-LonA1 and Mt-LonA2.Therefore, this study considered that Mt-TTC1975 did not exhibit the peptidase activity.

**Chaperone activity**

According to previous findings, Lon protease could serve as a chaperone (*34, 54*). To test if Mt-LonA1, Mt-LonA2 and Mt-TTC1975 possessed chaperone activity, this study examined whether its samples can prevent the aggregation of DTT-induced

denaturation of insulin. As shown in Fig. 5-17, DTT would induce the denaturation of insulin and formed aggregates, causing the insulin solution turbid. Contrastly, Mt-LonA1 and Mt-TTC1975 prevented the DTT-induced aggregation of insulin. For Mt-LonA2, this study detected a more turbid curve, suggesting that Mt-LonA2 did not prevent the DTT-induced aggregation of insulin, and Mt-LonA2 were denatured by DTT also. The result indicated that Mt-LonA1 and Mt-TTC1975 were efficient in preventing the aggregation of denatured insulin.

**DNA-binding activity**

To test the DNA-binding activity of Mt-LonA1, Mt-LonA2 and Mt-TTC1975, this study used the EMSA as described in Materials and Methods. According to previous studies, the α-domain of Lon protease was essential for DNA-binding activity (*55-56*), so that this study considered that Mt-LonA1 and Mt-LonA2 might exhibit the DNA-binding ability, but Mt-TTC1975 did not due to its lack of α-domain. As a result (Fig. 18A), Mt-LonA1 failed to bind DNA that was unexpected, and Mt-LonA2 showed a significant ability of DNA-binding. For Mt-TTC1975, this study found an expected result; Mt-TTC1975 failed to bind DNA. For positive control, Bt-Lon and Ec-Lon were used in these experiments as well (Fig. 5-18B). Bt-Lon and Ec-Lon showed lower DNA binding activity due to their lower concentrations.

# 4. Discussion

## 4-1. Phylogenetic analysis of Mt-LonA1, Mt-LonA2 and Mt-TTC1975

Lon proteases are a group of ATP-dependent serine proteases consisting of three functional domains: N-terminal domain, ATPase domain, and protease domain. Base on comparison of the sequences around the catalytic serine residue, many proteins were annotated to be Lon proteases. A total of 117 Lon protease homologues have been identified in the genome-wide survey study before (*20*). Many bacteria were discovered to have more than one Lon protease. For example, *T. thermophilus* HB27 has three proteases annotated to be Lon proteases (*39*). Two Lon proteases in *Borrelia burgdorferi* serve different functions (*57*). It appears that fewer research findings are yet available concerning this phenomenon. This study has sequenced the total genome of *Meiothermus taiwanensis* (WR-220) and found there are three proteases annotated as Lon in this bacterium, including Mt-LonA1, Mt-LonA2 and Mt-TTC1975.

Phylogenetic tree of various Lon proteases and TTC1975 peptidases (Fig. 5-5) showed that the LonA1 group and LonA2 group are obviously in different clades, and the graph (Fig. 5-5) reveals that the LonA2 group delegated from canonical LonA-type protease at early stage. Then, the remains split into LonA1 group and common LonA-type group. This phenomenon represents that the characteristics of LonA1 group

might be closer to the commonly LonA-type group, and the characteristics of LonA2 group might be slightly different from the precedents. This study considered that the LonA1 and LonA2 group of *thermus/meiothermus* genus can serve as different roles in the system of *thermus/meiothermus* genus. On the other hand, the TTC1975 peptidase group is distant from the groups mentioned above. Since TTC1975 peptidase lack intact Walker A and Walker B, it shall not be classified as a member of $AAA^+$ superfamily. In the past, the TTC1975 peptidase was annotated to be a Lon protease, yet recently Tomoko Maehara (*39*) has noticed a difference between TTC1975 peptidase and Lon protease. TTC1975 peptidase showed high homology with Lon protease in protease domain and conserved Ser-Lys dyad in the active site. This study considered that the evolutional relationship among LonA1, LonA2 and TTC1975 peptidase might have biological significance in *thermus/meiothermus* genus.

**4-2. Thermal stability of Mt-LonA1, Mt-LonA2 and Mt-TTC1975**

The stability of proteins is essential to the survival of thermophilic organisms at high temperature (*58*). To investigate the thermal stability of Mt-LonA1, Mt-LonA2 and Mt-TTC1975, this study uses turbidity assay to study thermal aggregation of these proteins. The results showed that Mt-LonA1 aggregates at 66 $^{o}$C, Mt-LonA2 at 70 $^{o}$C, and Mt-TTC1975 at 74 $^{o}$C (Fig. 5-9). This study also notices that the optimum growth

temperature of *Meiothermus taiwanensis* is approximately 55–60 °C (*3*). The melting temperature of Mt-LonA1, Mt-LonA2 and Mt-TTC1975 are higher than the optimum growth temperature of *Meiothermus taiwanensis*.

### 4-3. Structural characteristics of Mt-LonA1, Mt-LonA2 and Mt-TTC1975

The Lon proteases are members of the ATP-dependent proteases of $AAA^+$ superfamily who possess the oligomeric structure which required for their activities. Regarding the N-terminal domain, Shrader and Roudiak (*59*) have demonstrated how the three N-terminal truncations of Lon protease from *Mycobacterium smegmatis* (Ms-Lon), while lacking 90, 225, and 277 residues (named as Ms-Lon N-G91, N-E226, and N-I278, respectively), reveal different behaviors toward their functions and quaternary structures. For instance, Ms-Lon N-G91 and N-E226 retain peptidase activities and basal ATPase activities, and their quaternary structures are not altered drastically. Ms-Lon N-I278, however, displays neither peptidase nor ATPase activity and behaves as a higher multi-meric state despite the fact that it is stable and soluble in solution. Furthermore, the N-terminal domain of Lon protease from *Brevibacillus thermoruber* deletes more residues (named as Bt-LonΔN) (*55*), and the mutant, Bt-LonΔN, is with the deletion of 316 residues in N-terminal defects in oligomerization. These results indicate that the N-terminal domain is essential for oligomerization, and

the quaternary structure characteristics are important for activities of Lon protease. To investigate the quaternary structure of Mt-LonA1, Mt-LonA2 and Mt-TTC1975, This study first examines the secondary and tertiary structure of them. The CD spectra of Mt-LonA1, Mt-LonA2 and Mt-TTC1975 indicate that they posses well-define three dimensional structures (Fig. 5-7, 5-8). For quaternary structure, the AUC data, Native-PAGE and EM graph reveal that Mt-LonA1 functions mainly as a hexamer; the AUC data and Native-PAGE reveal that Mt-LonA2 might function as a mixture of dimer and pentamer; Native-PAGE reveals that Mt-TTC1975 functions as a hexamer or a heptamer (Fig.5-10, 5-12, 5-13). Although This study have known through the collected information, it still lacks direct evidence to prove whether the N-terminal domains are essential for oligomerization of Mt-LonA1, Mt-LonA2 and Mt-TTC1975, and whether the quaternary structures of Mt-LonA1, Mt-LonA2 and Mt-TTC1975 are essential for their activities.

**4-4. Catalytic activity of Mt-LonA1, Mt-LonA2 and Mt-TTC1975**

The catalytic activities (including ATPase, protease and peptidase) of Lon protease are dependent upon their tertiary and quaternary structures (*60-62*). To identify the catalytic activities, This study chooses the ideal growing temperature 55°C of *Meiothermus taiwanensis* for this purpose. The catalytic activities results have been

summarized in Table. 6-3. From the summary, one can see that Mt-LonA1 and Mt-LonA2 both exhibit the ATPase , protease and peptidase activities, but Mt-TTC1975 does not. The results can be considered predictable, because Mt-LonA1 and Mt-LonA2 both possess the classical LonA-type domains including N-terminal, ATPase, and protease domain. For Mt-TTC1975, it only possesses a high similarity in protease domain with canonical LonA. Thus, this study considers the results are reasonable for Mt-TTC1975.

## 4-5. Characterization of chaperone activity of Mt-LonA1, Mt-LonA2 and Mt-TTC1975

Lon had been proven to have chaperone activity (*55, 63-66*). In this study, the results also prove that Mt-LonA1 and Mt-TTC1975 possess the chaperone activity but Mt-LonA2 does not (Fig. 5-17). Mt-LonA1 and Mt-TTC1975 show chaperone activity in an ATP-independent manner. As the results, Mt-LonA1 and Mt-TTC1975 are bound to the denatured insulin (induced by DTT) and prevent the aggregation of denatured insulin. This study can consider that Mt-TTC1975 might function like α-crystallin, a known molecular chaperone which functions in an ATP-independent manner (*67-68*).

**4-6. DNA-binding and α-domain**

Lon had been known as a DNA-binding protein as well. In this study, the structure-functional relationship is proposed. According to previous findings, the α-domain is the key domain of DNA-binding (*55, 66*). Based on these inspiring findings, the structural information of α-domain has been revealed further (*69*), due to the improvement of NMR technique. Furthermore, the positively charged surface of the α-domain from Bt-Lon has been pointed out that the R518 residue of α-domain might critically influence the DNA-binding activity (*56*).

The three putative Lon proteases from *Meiothermus taiwanensis* (WR-220), Mt-LonA1, Mt-LonA2 and Mt-TTC1975 are being investigated, and the ability of DNA-binding is shown as above. From the current research results (Fig. 5-18A), Mt-LonA1 does not show the ability of DNA-binding that is unexpected because of its existence of α-domain. At the same time, Mt-LonA2 shows the ability of DNA-binding strongly that has been expected because of its existence of α-domain. For Mt-TTC1975, due to its lack of α-domain, an expected result comes out that the Mt-TTC1975 does not bind to the DNA. Owing to the results, this study contemplates on why the Mt-LonA1 does not exhibit the DNA-binding activity. For this purpose, this study compares the α-domain sequences of Mt-LonA1, Mt-LonA1 and Bt-Lon (Fig. 5-19). As a result, Mt-LonA1 and Mt-LonA2 both possess glutamate residue at the relative position toward

Bt-Lon R518 residue, meaning that Mt-LonA2 might possess another important positively charged residue near the conserved region. Interestingly, a lysine residue is found, and a positively charged residue is located on the position just before the glutamate residue. Furthermore, there is a conserved region between Mt-LonA2 and Bt-Lon that has been prevalent in positively charged amino acid residues. The K-K-R conserved region is underlined in Fig. 5-19 and pointed in Fig. 5-20. The K527 residue and the K-K-R conserved region of Mt-LonA2 might critically influence the DNA-binding activity. Nevertheless, the conclusion still needs more evidence to prove the inference.

Lastly, this study constructs the graph showing the homology modeling of α-domains (including the α-domain of Mt-LonA1, Mt-LonA2, Bt-Lon and Ec-Lon). While the Mt-LonA2, Bt-Lon and Ec-Lon all posses the ability of DNA-binding, Mt-LonA1 does not. Hence, the distribution of positively charged residues on the surface of the top view from α-domains might critically influence the ability of DNA-binding.

## 5. Figure

**Mt-LonA1 :**

CAGACCCATGACAGCCCTGGAGGCCGGGTATAACCTGGGGTGGCGGGCCAAACTTGAGTG

AGATTTACTCAAGTTCGCTAAGTGTAAGATTTGACCCTATCGGTGAACCATCACCGTTTAGA

ATCTGAGGTATATATCGCTCCCATACCGCCGCGAAGCGACGAATCATAGGCGGAACATAGG

A**GATAGATA**CGATATG

**M**

**Mt-LonA2 :**

CCACGGCCCAGAGCAACAGTAGAACCCCAATCCGGGTAAGCCTCATCTACTTCAGCATACGG

TATTGTGGTGTGGGTTCTTATTGTTATTGATACAATCACTAAACTTGATAATAATAGACTCAGG

GTGTTGACTCACACACCCGTCCACACAAAGCCTGCGCTGCCTGTACACTTGC**GATAGATA**GG

GAGTTATTTATG

**M**

**Figure 5-1. Putative promoter region of Mt-LonA1 and Mt-LonA2**

Potential promoter region are underlined. The filled circles indicate the transcription start site. The GATA transcription elements are boxed near the -10 region.

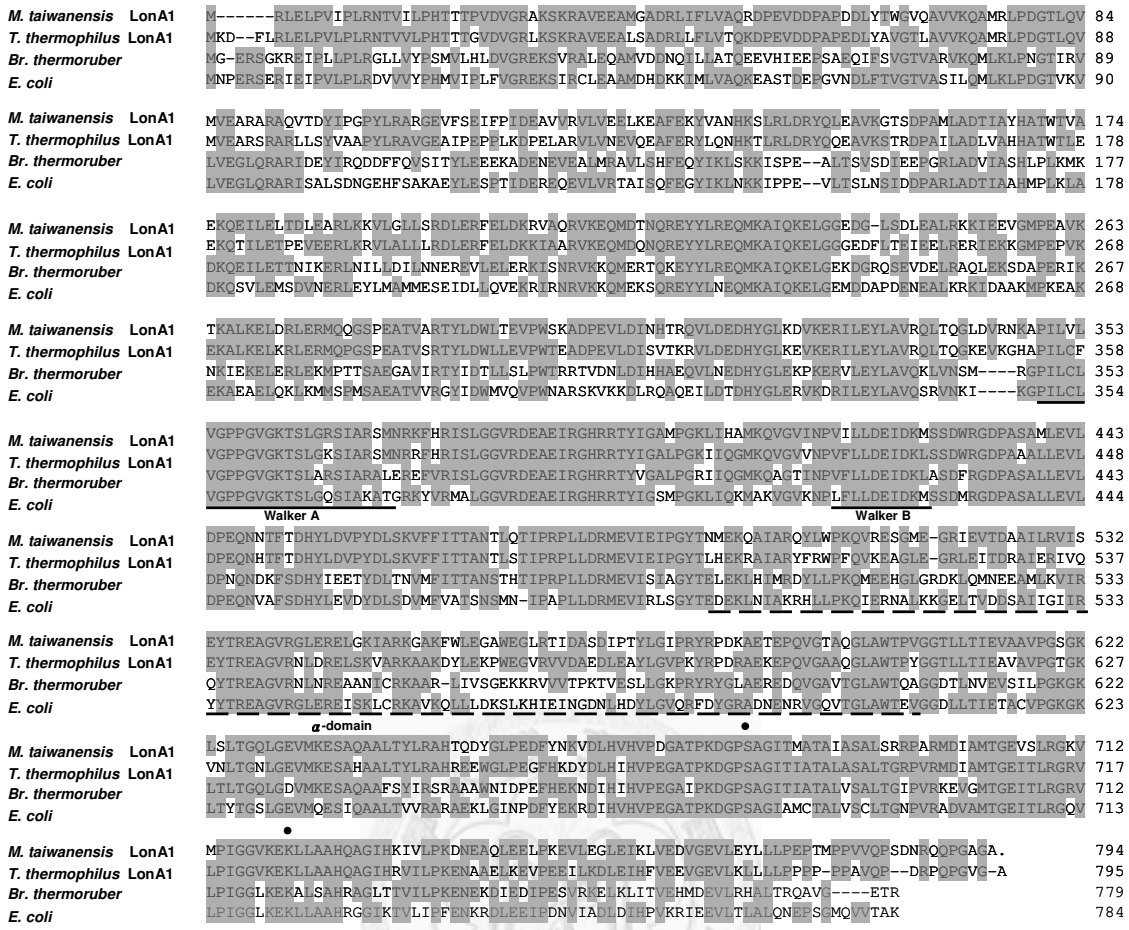



**Figure 5-2. The amino acid sequence alignment of Mt-LonA1 and other Lon protease**

Identical amino acid residues are shaded. The broken underline indicates the conserved α-domain. The filled circles indicate the Ser-Lys dyad of the protease active site.

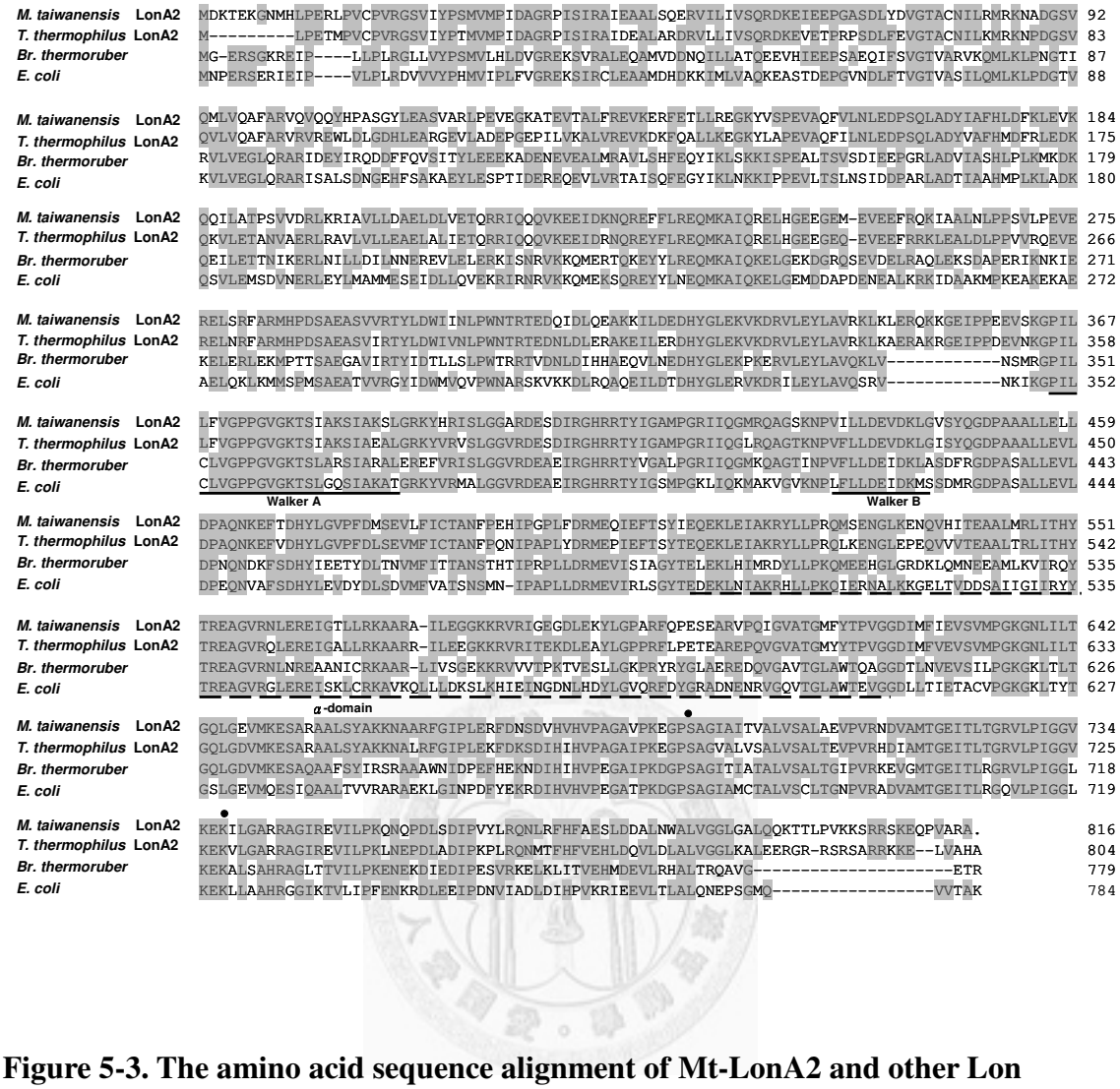



**Figure 5-3. The amino acid sequence alignment of Mt-LonA2 and other Lon protease**

Identical amino acid residues are shaded. The broken underline indicates the conserved α-domain. The filled circles indicate the Ser-Lys dyad of the protease active site.


**Figure 5-4. The amino acid sequence alignment of Mt-LonA1 and other Mt-LonA2**

Identical amino acid residues are shaded. The broken underline indicates the conserved α-domain. The filled circles indicate the Ser-Lys dyad of the protease active site.

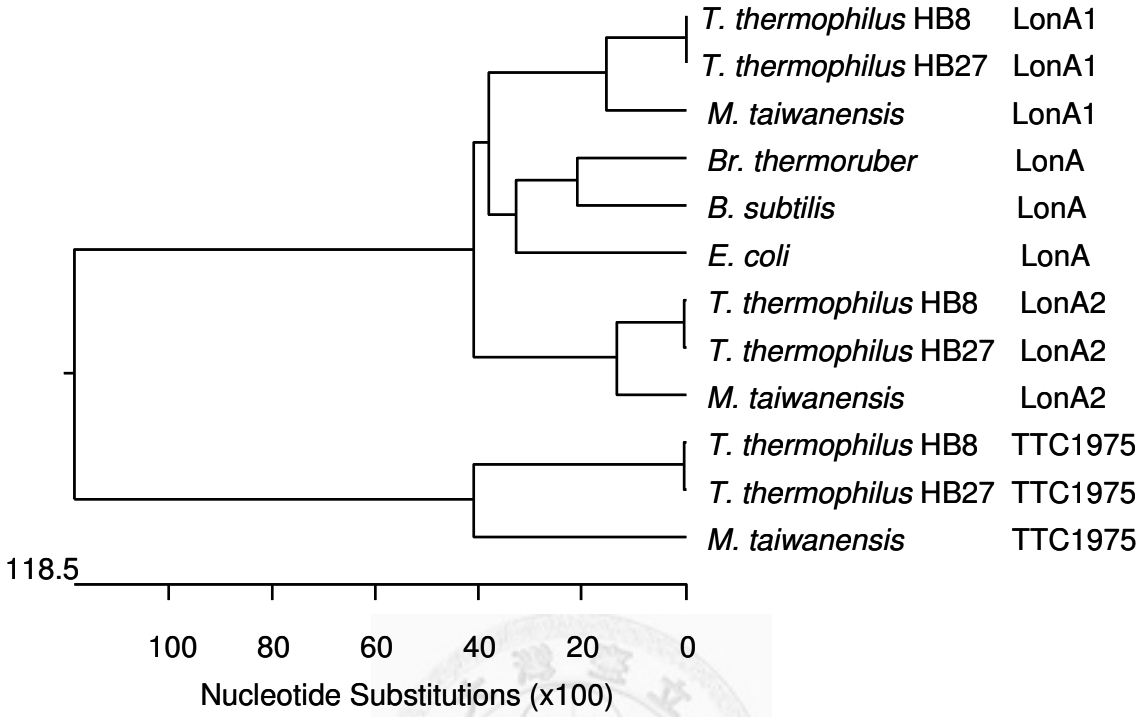



**Figure 5-5. The phylogenetic tree of Lon proteases**

The diagram was produced by program DNAstar MegAlign (phylogenetic tree). The species that were compared include *Meiothermus taiwanensis, T. thermophilus, Br. Thermoruber, B. subtilis,* and *E. coli.*

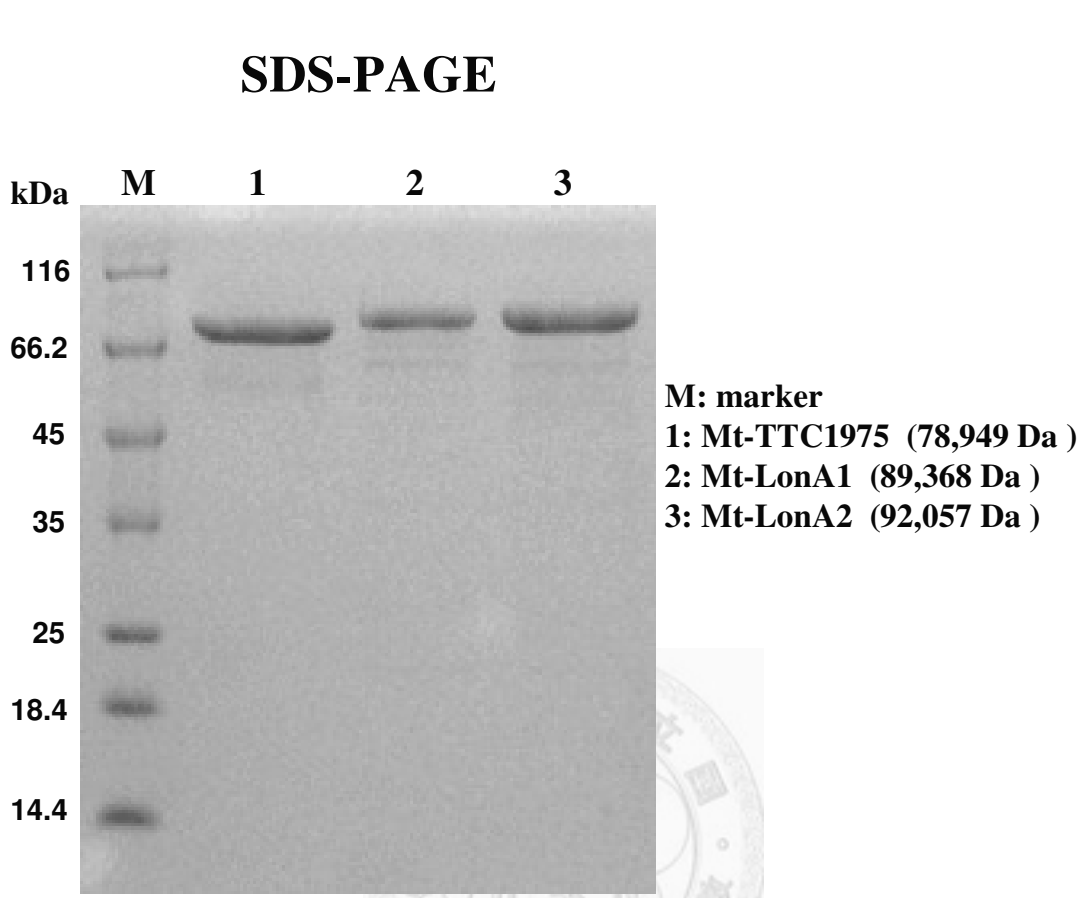



**Figure 5-6. SDS-PAGE of purified recombinant Mt-LonA1, Mt-LonA2 and Mt-TTC1975**

PAGE was stained by coomassie brilliant blue. The predicted molecular masses of His-tagged Mt-LonA1, Mt-LonA2 and Mt-TTC1975 are 89,368 Da, 92,057 Da and 78,949 Da, respectively.

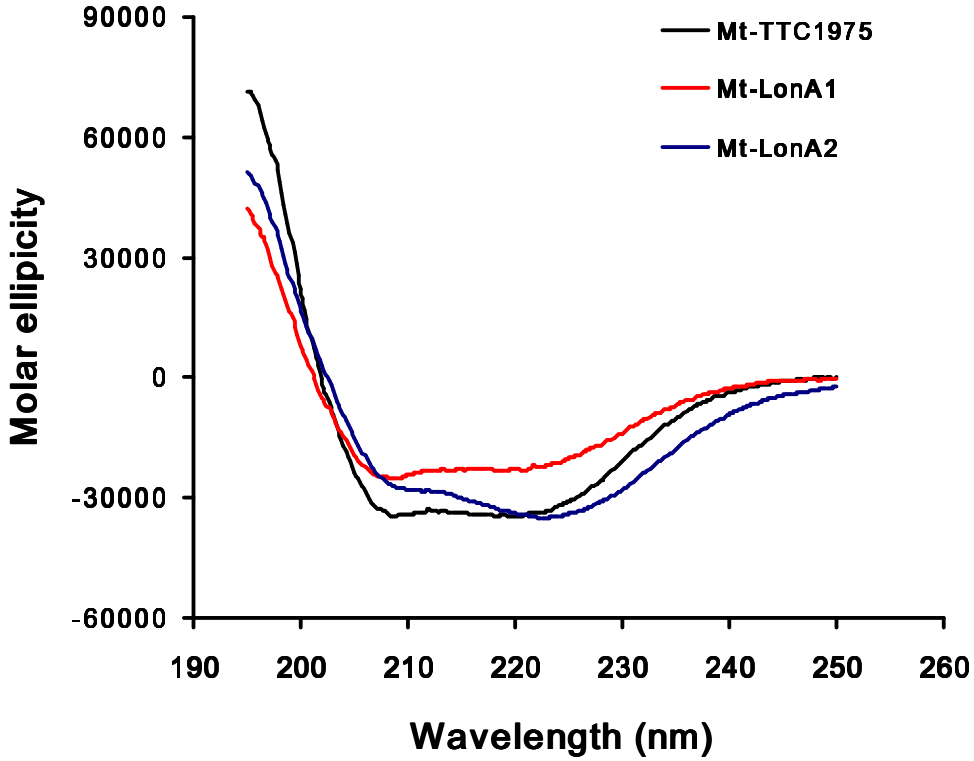



**Figure 5-7. Far-UV CD spectra**

Protein concentrations were 0.7 μM. The protein samples were scanned from 250 to 200 nm at 25 $^{o}$C. Data is presented as molar ellipticity (deg cm$^{2}$ dmol$^{-1}$).

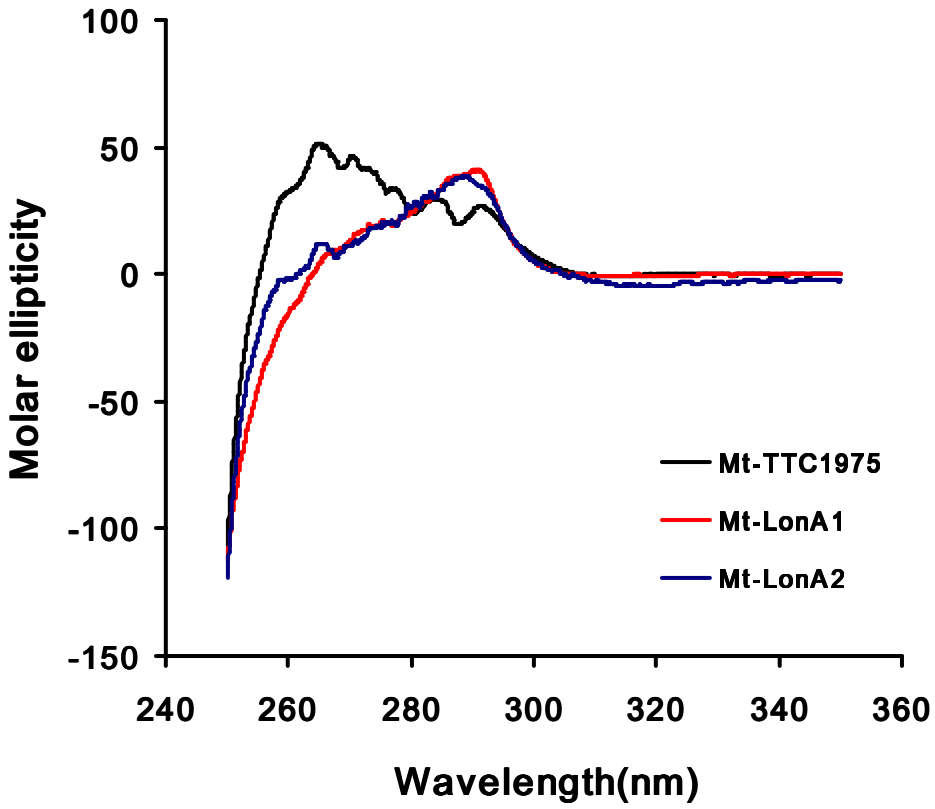



**Figure 5-8. Near-UV CD spectra**

Protein concentrations were 20 μM. The protein samples were scanned from 350 to 250 nm at 25 $^{o}$C. Data is presented as molar ellipticity (deg cm$^{2}$ dmol$^{-1}$).

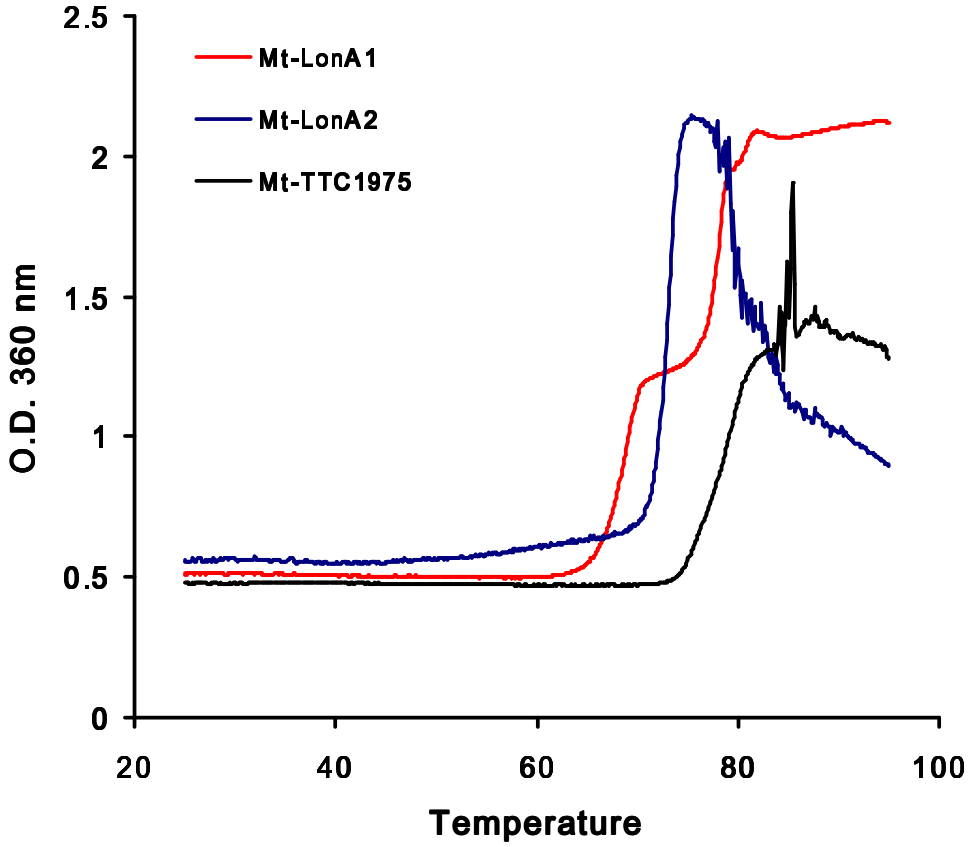



**Figure 5-9. Thermostability of Mt-LonA1, Mt-LonA2 and Mt-TTC1975**

The turbidity of Mt-LonA1, Mt-LonA2 and Mt-TTC1975 were measured at temperature higher than 66 °C, 70 °C and 74 °C, respectively. The results indicated the melting temperature respectively.

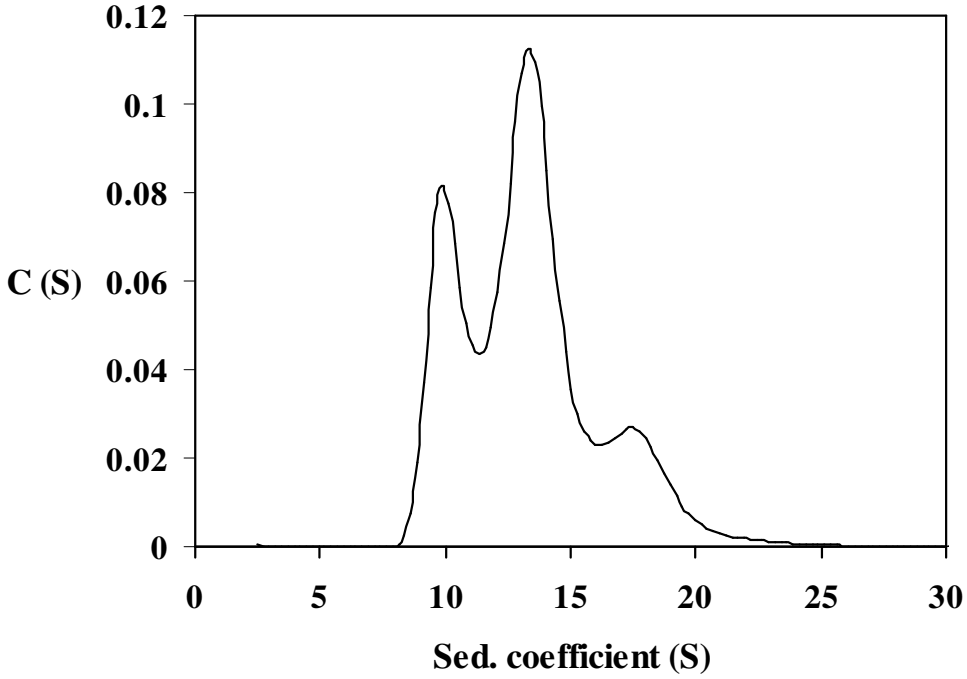



**Figure 5-10. Sedimentation velocity experiment of Mt-LonA1**

Sedimentation velocity was measured at 20,000 rpm with standard double-sector centerpieces at 20 $^{o}$C. The UV absorption (280 nm) of the cells was scanned every 4 min for 250 scans. The data was analyzed with popular software SEDFIT. The sample was diluted to 0.5 in $OD_{280}$ (10 μM) with PBS buffer (pH 7.4) containing 10% glycerol and 5 mM $MgCl_2$, and was visually checked for clarity after.

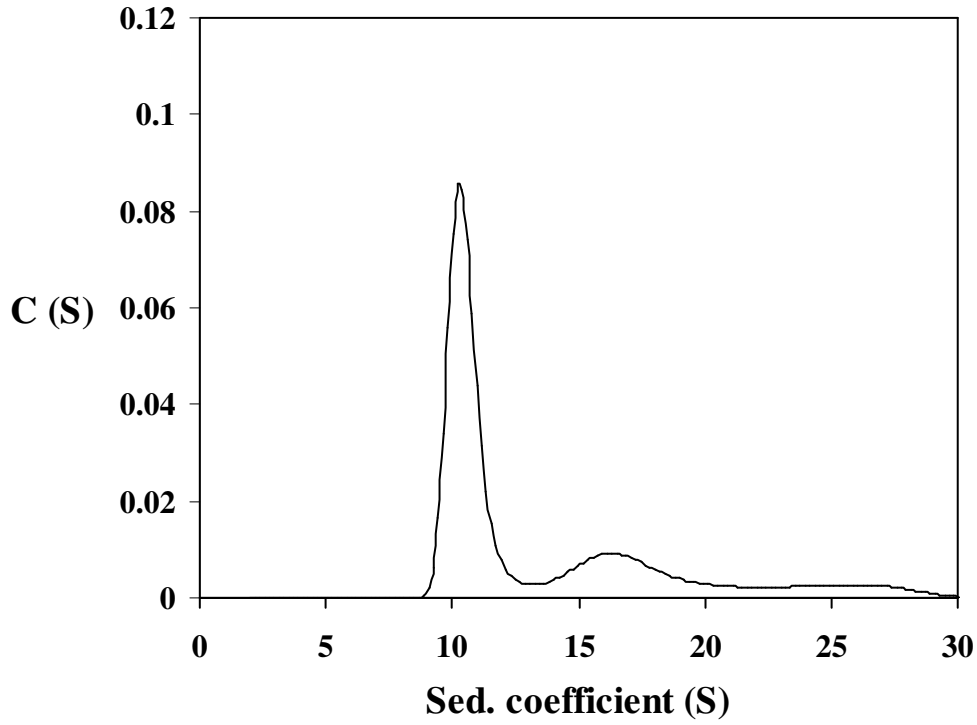



**Figure 5-11. Sedimentation velocity experiment of Mt-LonA2**

Sedimentation velocity was measured at 20,000 rpm with standard double-sector centerpieces at 20 $^{o}$C. The UV absorption (280 nm) of the cells was scanned every 4 min for 250 scans. The data was analyzed with popular software SEDFIT. The sample was diluted to 0.5 in $OD_{280}$ (1 μM) with PBS buffer (pH 7.4) containing 10% glycerol and 5 mM $MgCl_2$, and was visually checked for clarity after.

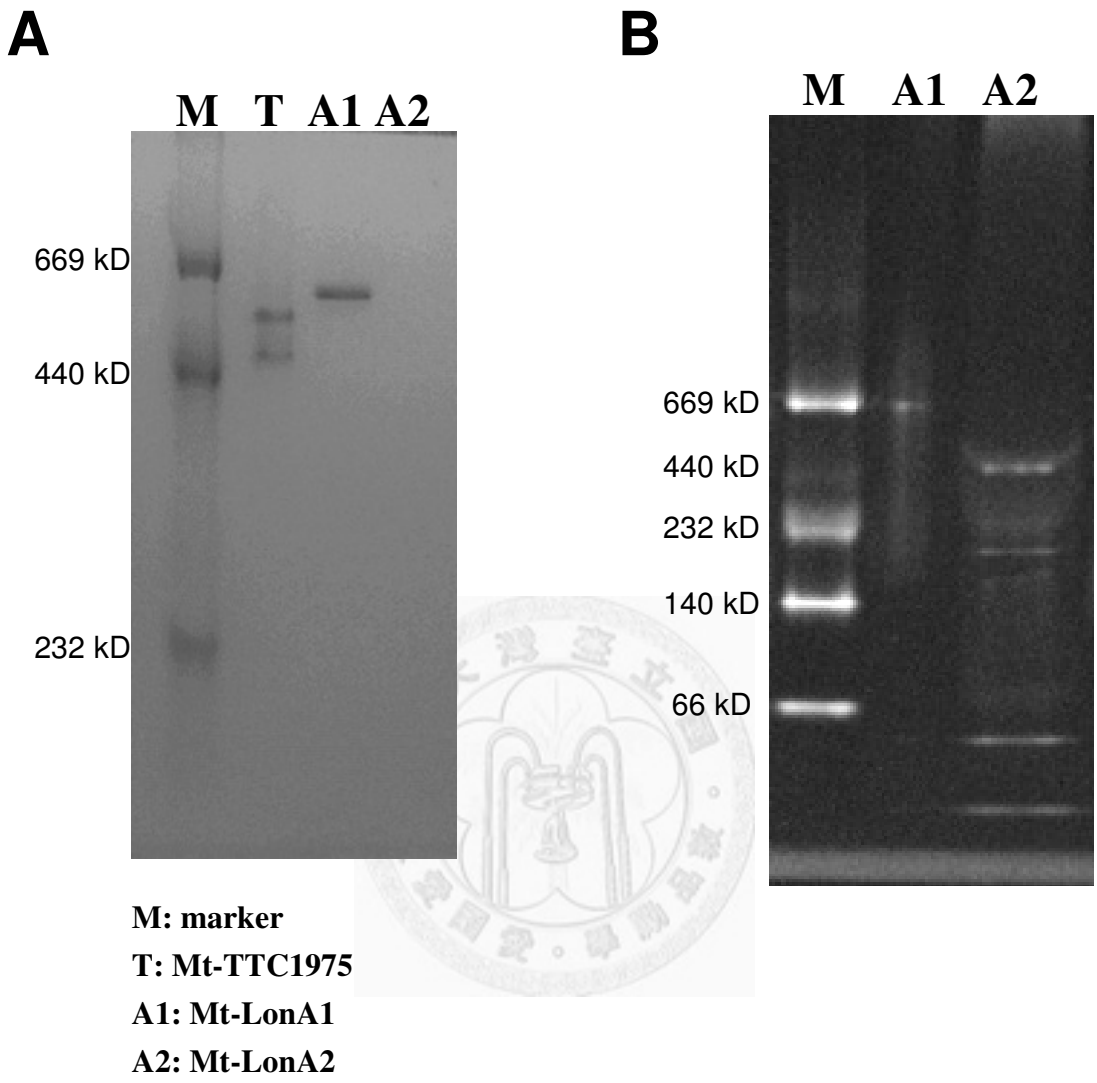



**Figure 5-12. Native PAGE of Mt-LonA1, Mt-LonA2 and Mt-TTC1975**

4 μg of samples were loaded in every well respectively. (A) Coomassie brilliant blue staining. (B) SYPRO-Ruby staining

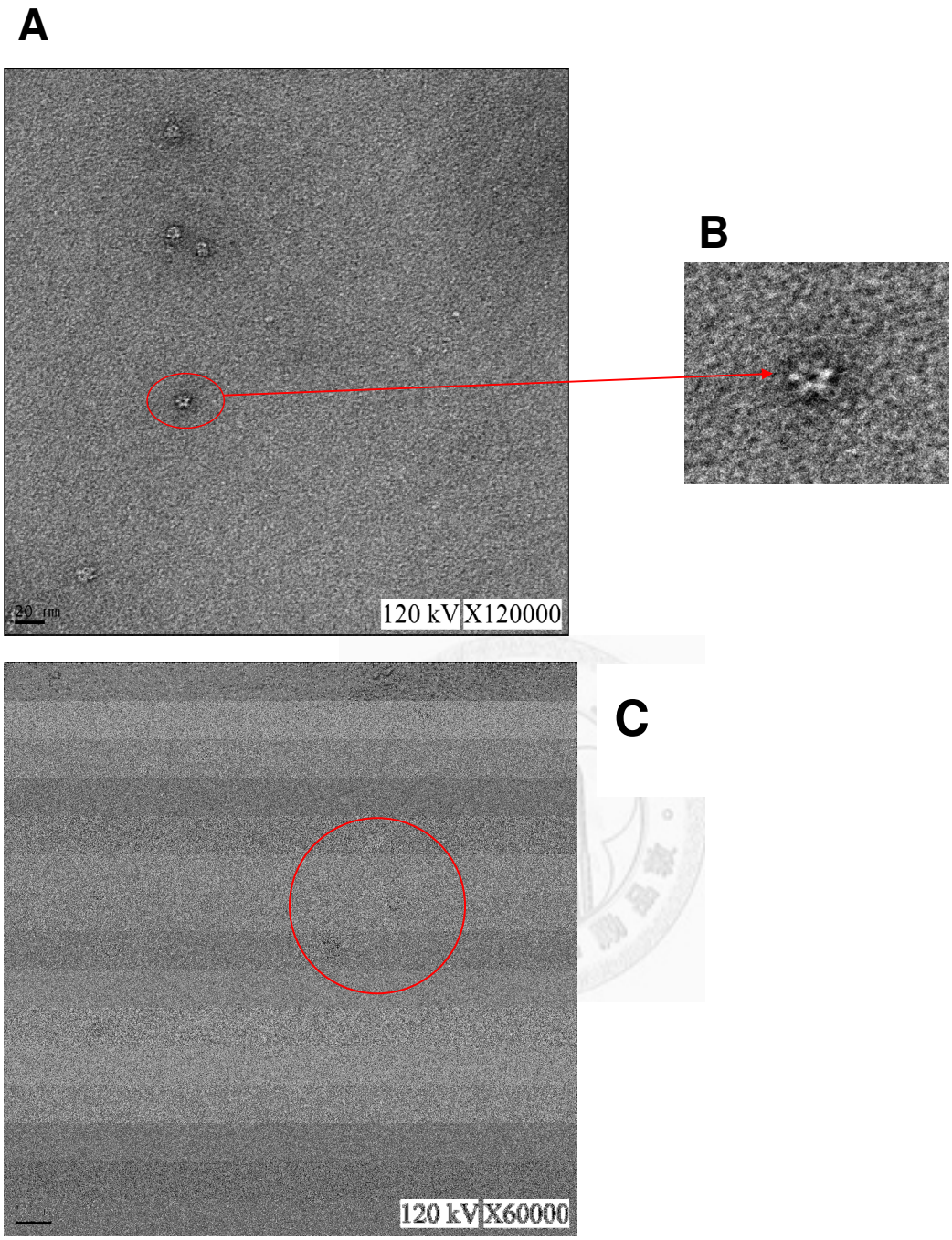



**Figure 5-13. Transmission electron microscopy images of Mt-LonA1**

(A) Negative staining with uranyl acetate showed four regular ring-shaped particles. (B) The hexamer of Mt-LonA1. (C) A wider view of negative staining EM showed some larger particles that consist of three or two hexamer of Mt-LonA1.

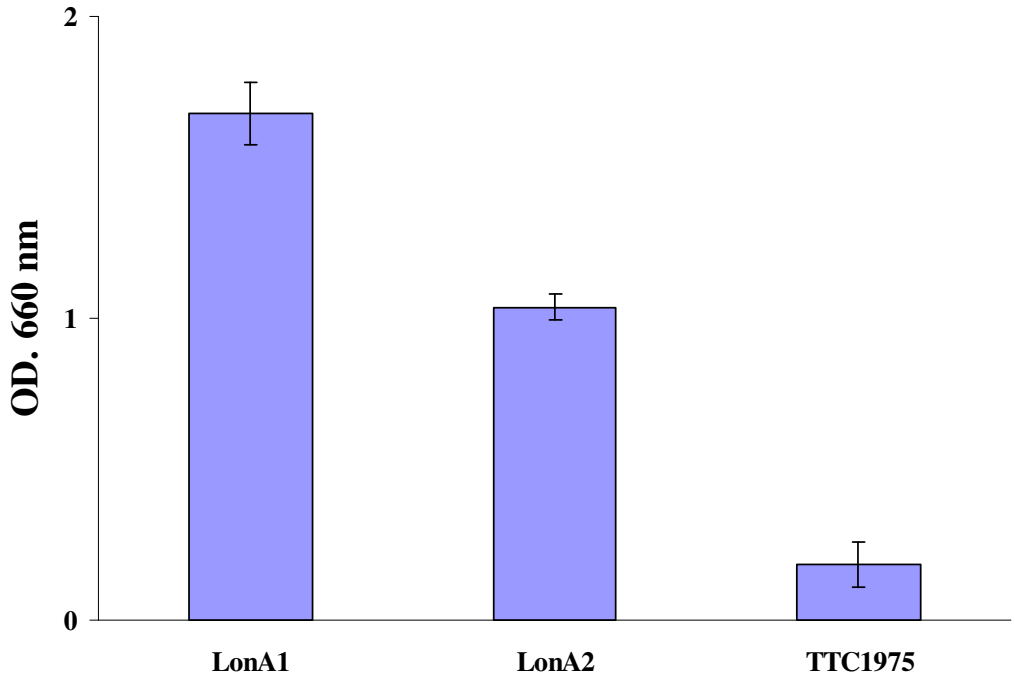



**Figure 5-14. ATPase activity assay**

The absorbance (660 nm) of malachite / molybdate solution indicates the level of ATPase activity. The sample mixtures were incubated at 55 $^{o}$C for 30 minutes.

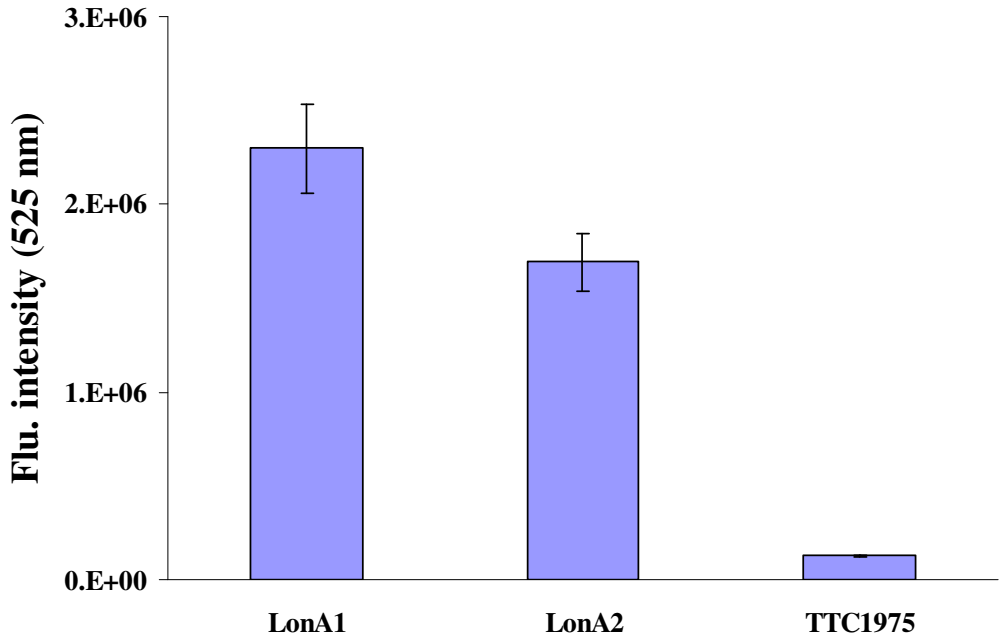



**Figure 5-15. Protease activity assay**

FITC-α-casein was used as a substrate to react with Mt-LonA1, Mt-LonA2 and Mt-TTC1975 in the presence of ATP at 55 $^{o}$C for 60 minutes.

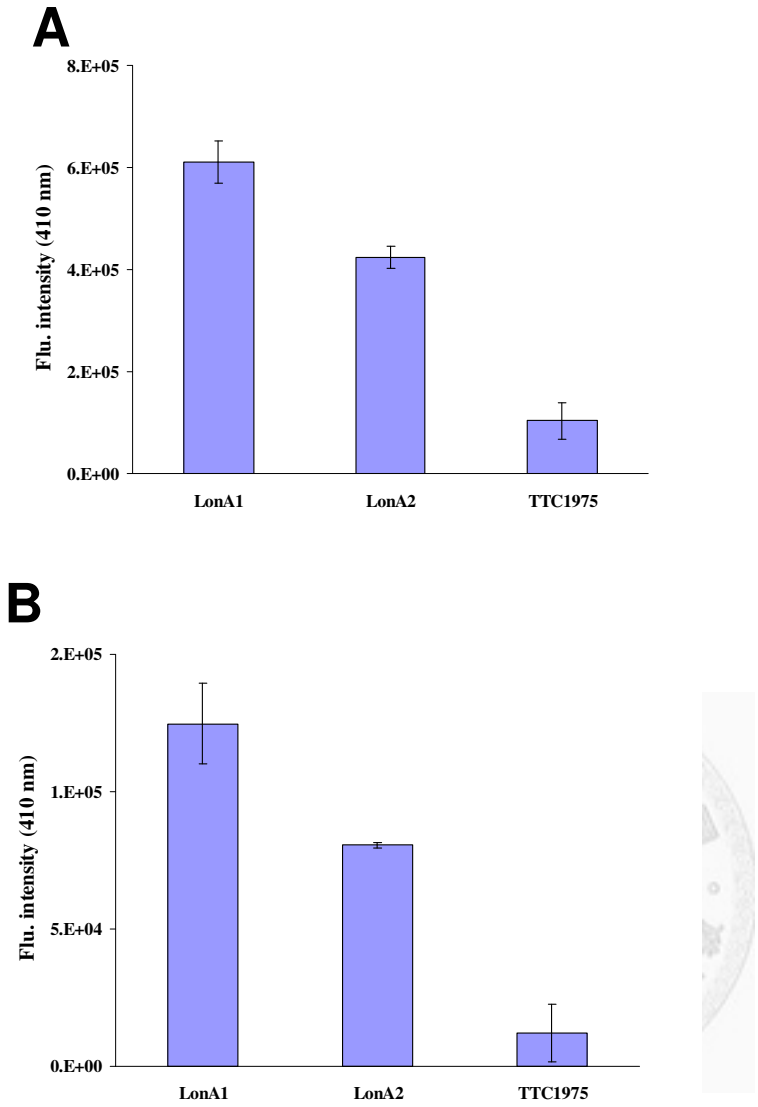
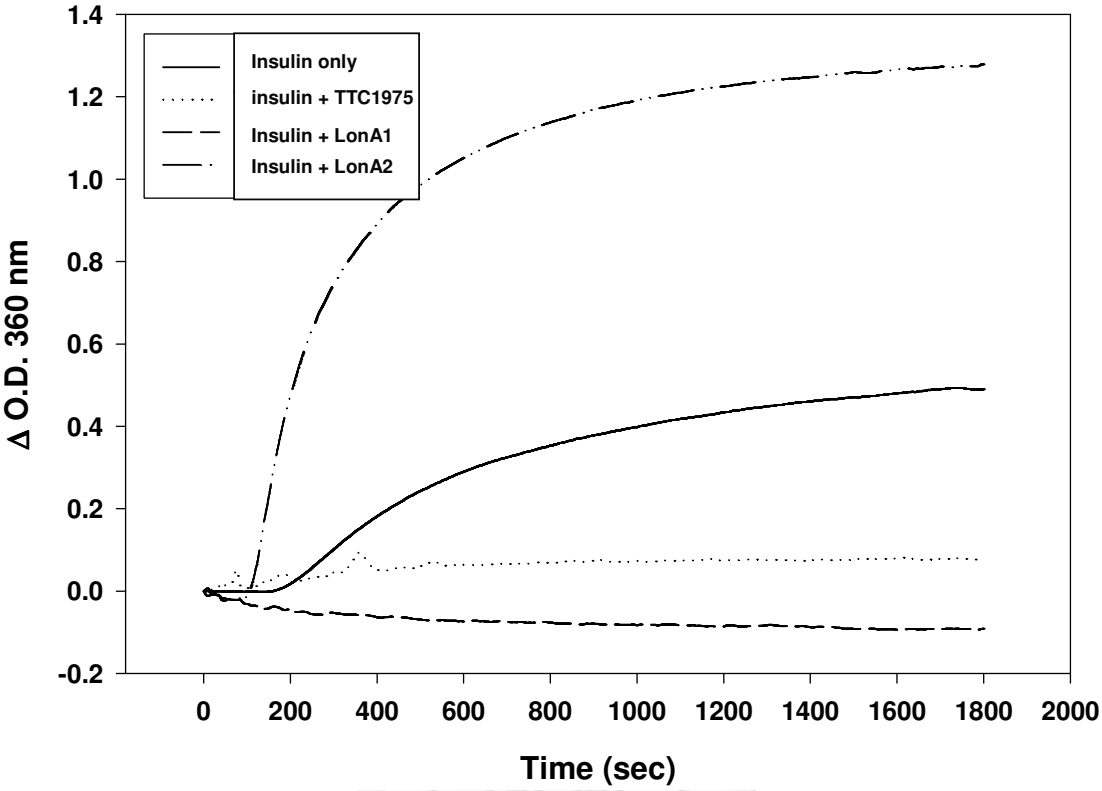



**Figure 5-16. Peptidase activity assay**

(A) Fluorogenic peptide (Glt-AAF-4MNA) was used as a substrate to react with Lon proteases sample in the presence of ATP at 55 $^{o}$C for 60 minutes. (B) Fluorogenic peptide (Suc-FLF-4MNA) was used as a substrate and the experimental condition was same as (A).




**Insulin: 52.4 μM**
**LonA1,LonA2: 13.1 μM**
**TTC1975:52.4 μM**
**DTT: 20 mM**
**Temperature: 37℃**

**Figure 5-17. Chaperone activity assay**

Bovine insulin (52.4 μM) in PBS buffer at pH 7.4 was unfolded by adding DTT to reach 20 mM as the final concentration at 37 °C. The protein concentrations were 13.1 μM for Mt-LonA1and Mt-LonA2, and 52.4 μM for Mt-TTC1975.

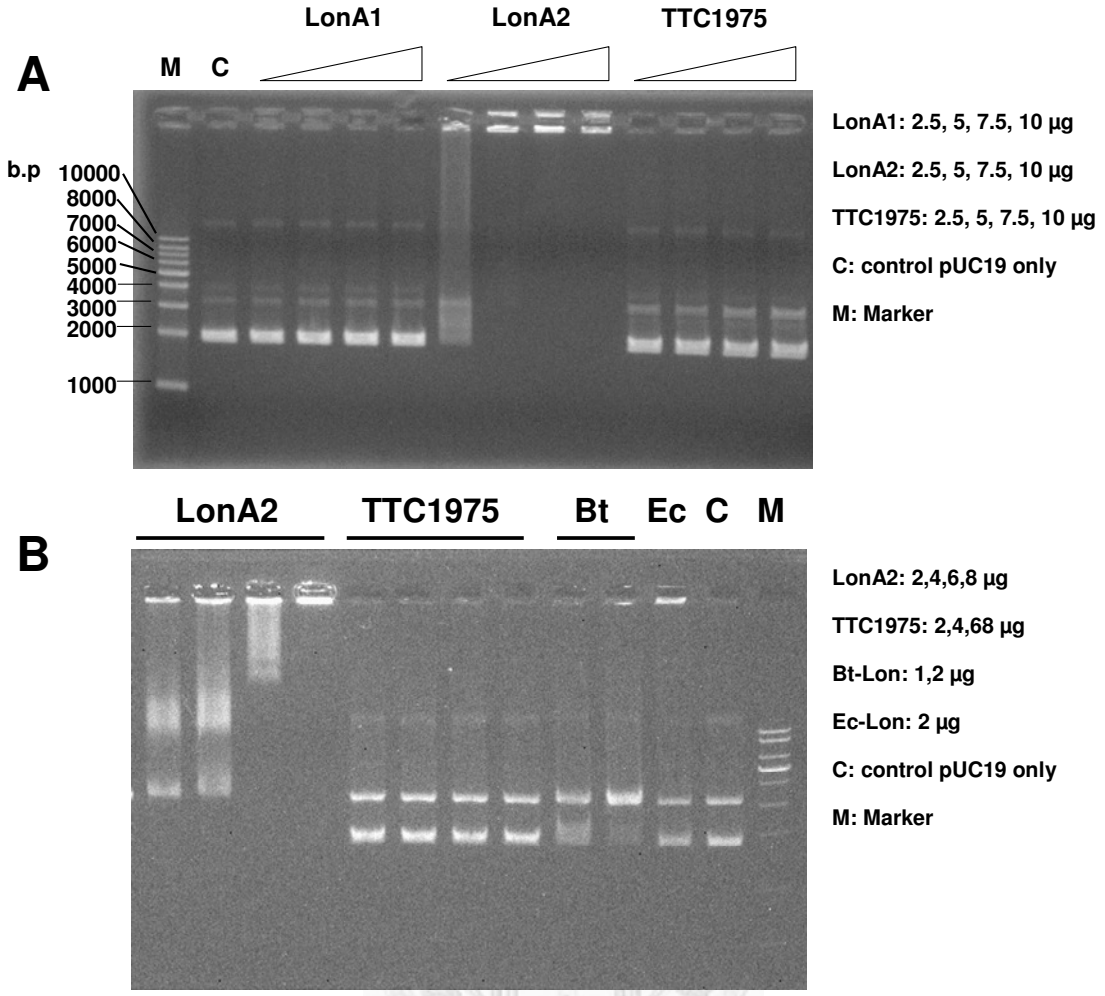



**Figure 5-18. Electrophoretic mobility shift assay**

(A) Plasmid DNA pUC-19 was used as a substrate for Mt-LonA1, Mt-LonA2 and Mt-TTC1975. The pUC-19 is a 2,686 b.p. circular plasmid DNA. The control showed that pUC-19 had a major super-coiled form near the 2,000 b.p. marker and a minor open circular form near the 3,000 b.p. marker in this experiment. All Reaction mixtures were incubated at 55 $^{o}$C for 5 minutes. (B) Bt-Lon and Ec-Lon were use as positive controls. Bt-Lon and Ec-Lon showed lower DNA binding activity due to their lower concentrations.

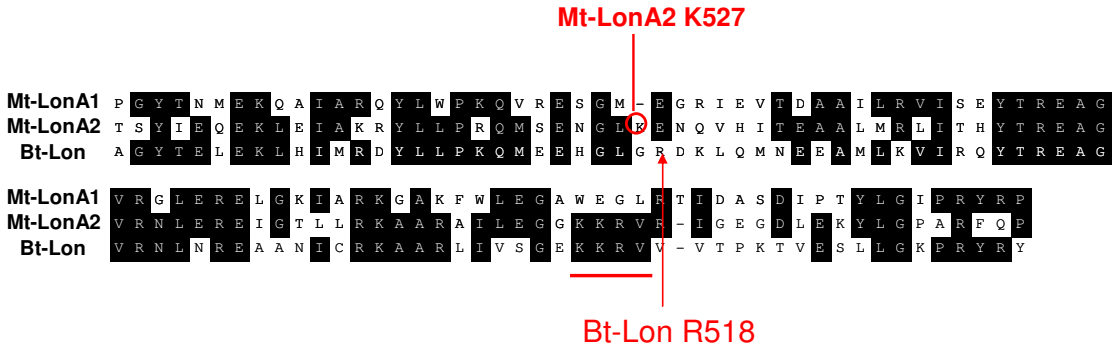



**Figure 5-19. Amino acid sequence alignment of α-domains**

The alignment was generated using the program DNAstar MegAlign (ClustalV Method). The original sequences used in the analysis: Mt-LonA1, residues 490-585; Mt-LonA2, residues 501-607; Bt-Lon, residues 491-585.

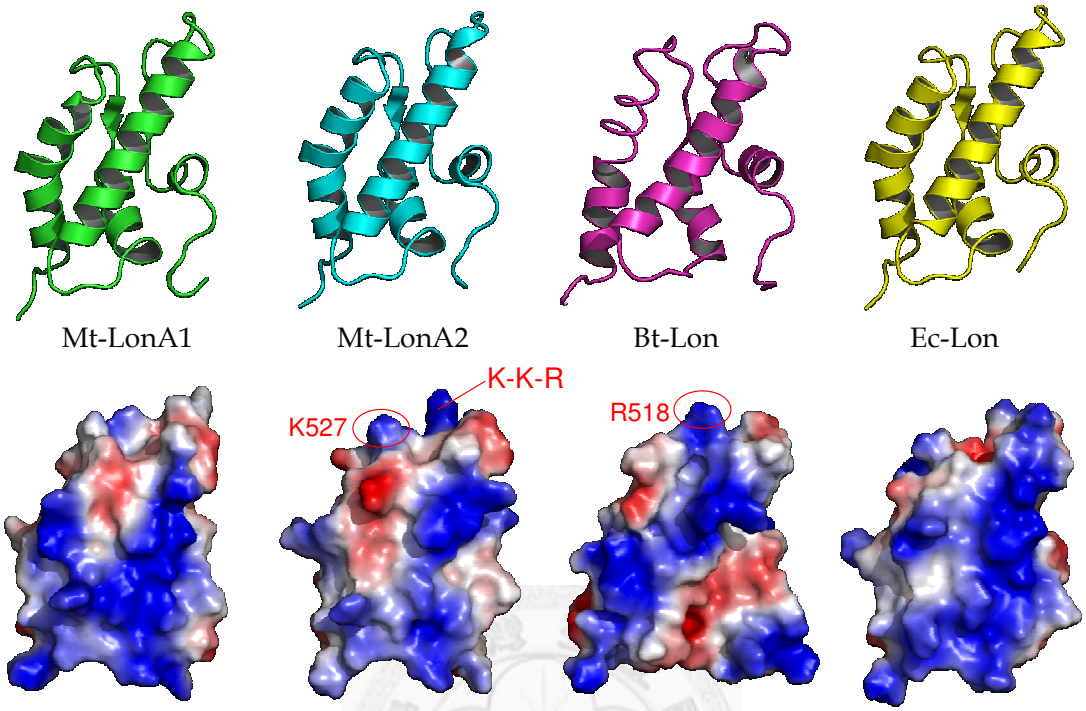



**Figure 5-20. Homology modeling of α-domains**

Comparison of the surface structures of Mt-LonA1, Mt-LonA2, Bt-Lon and Ec-Lon α-domain. Positive and negative charges are shown in blue and red, respectively.

# 6.Table

**Table 6-1**

| Primers for cloning | |
|---|---|
| LonA1-F | 5’- CATATGCGTTTAGAACTGCCGGT- 3’ |
| LonA1-R | 5’- AAGCTTTGCCCCAGCGCCA - 3’ |
| LonA1-Mid-F | 5’- GGCCCTGCGCAAAAAGATCGAAGA - 3 |
| LonA1-Mid-R | 5’- CGGTAGCGGGGGATACCCAGATAA - 3’ |
| LonA2-F | 5’- GGAATTCCATATGGACAAAACCGAAAAAGGC - 3’ |
| LonA2-R | 5’- CCCAAGCTTGGCCCTGGCTACA - 3’ |
| LonA2-Mid-F | 5’- GCGAGCTTCACGGCGAAGAGG - 3’ |
| LonA2-Mid-R | 5’- TGTAGAACATTCCCGTAGCCACC - 3’ |

**Table 6-2**

**The secondary structure ratios of Mt-LonA1, Mt-LonA2 and Mt-TTC1975**

| | Method | Helix( r ) | Helix( d ) | Strand( r ) | Strand( d ) | Turn | Unorder |
|---|---|---|---|---|---|---|---|
| Mt-LonA1 | Selcon3 | 0.617 | 0.219 | 0.022 | 0.009 | 0.173 | 0.14 |
| | Cdsstr | 0.534 | 0.254 | 0.051 | 0.046 | 0.044 | 0.081 |
| | Continll | 0.784 | 0.195 | 0 | 0.022 | 0 | 0 |
| Mt-LonA2 | Selcon3 | 0.579 | 0.227 | 0.004 | 0.003 | 0.051 | 0.14 |
| | Cdsstr | 0.669 | 0.281 | 0.03 | 0.007 | 0.012 | 0.015 |
| | Continll | 0.644 | 0.278 | 0.004 | 0 | 0.006 | 0.068 |
| Mt-TTC1975 | Selcon3 | 0.587 | 0.217 | 0.001 | 0.002 | 0.048 | 0.141 |
| | Cdsstr | 0.486 | 0.306 | 0.062 | 0.038 | 0.069 | 0.05 |
| | Continll | 0.72 | 0.267 | 0.002 | 0 | 0 | 0.011 |

**r: regular**

**d: distorted**

**Table 6-3**

**Summary of Mt-LonA1, Mt-LonA2 and Mt-TTC1975**

| | ATPase | Protease | Peptidase | Chaperone | DNA-binding | Quaternary structure |
|---|---|---|---|---|---|---|
| **Mt-LonA1** | ○ | ○ | ○ | ○ | X | **Hexamer** |
| **Mt-LonA2** | ○ | ○ | ○ | X | ○ | **Dimer; Pentamer** |
| **Mt-TTC1975** | X | X | X | ○ | X | **Hexamer; Heptamer** |

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