UNIVERSITI PUTRA MALAYSIA

# SYNTHESIS AND CHARACTERIZATION OF SEMISYNTHETIC METALLOTHERMOLYSIN

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FS 2008 17

# SYNTHESIS AND CHARACTERIZATION OF SEMISYNTHETIC METALLOTHERMOLYSIN

**By**

**SYARAJATUL ERMA KHALID**

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirement for the Degree of Master of Science.

April 2008

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**MASTER OF SCIENCE**
**UNIVERSITI PUTRA MALAYSIA**

**2008**

Abstract of the thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Master of Science

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**Chairman : Associate Professor Mohd. Basyaruddin Abdul Rahman, PhD**

**Faculty : Science**

Modification of native enzyme has gone through various strategies and evolution of the process itself. Thermolysin (TLN) from *Bacillus thermoproteolyticus rokko* was modified as a semisynthetic metallothermolysin which comprises enzyme, ligand and metal ion. Ligands used were benzamidine (BEN), 1,10-phenanthroline (PHN), *p*-aminobenzamidine (PBZ) and ethanolamine (ETA). The metal ions chosen were magnesium ($Mg^{2+}$), zinc ($Zn^{2+}$), calcium ($Ca^{2+}$) and nickel ($Ni^{2+}$). The semisynthetic metallothermolysin activities were evaluated on hydrolysis reaction of azocasein. Among the four ligands, complex of TLN-PBZ showed the highest specific activity (2219.5 Unit per mg (U/mg)) at optimum PBZ concentration of 0.6 mM. The study followed by the attachment of $Mg^{2+}$ to TLN-PBZ complex which gave the best specific activity compared to other metal ions (39406.4 U/mg). The optimum concentration of $Mg^{2+}$ was found

best at 0.08 mM. Several parameters were also investigated such as studies on effect of pH, temperature, time course and thermostability. As a result, the semisynthetic metallothermolysin maintained at pH surrounding of 7.0 in tris-HCl buffer and found optimum at 80$^{o}$C for reaction up to 3 hours (96.7% of relative activity). For thermostability test, the semisynthetic metallothermolysin can retain its activity up to 90% at pre-heated temperature of 80$^{o}$C.

Electronic absorption like the UV/Visible (UV/Vis) and UV/Fluorescence spectrophotometer and Circular Dichroism (CD) spectropolarimetry method were used to characterize the optical properties of metallothermolysin. In UV/Vis spectrophotometer, the binding of PBZ to TLN curve caused a bathocromic shift ($\lambda_{max}$ from 279 nm to 274 nm) and became hypsochromicsm ($\lambda_{max}$ from 274 nm to 272 nm) with the additional of $Mg^{2+}$. Changes in UV/Vis were also supported by UV/Fluorescence, when changes happened to the emission characteristic of TLN-PBZ spectrum (373.2 nm) and the spectrum continues to shift (374.0 nm) for TLN-PBZ-Mg. The CD spectropolarimetry suggested some changes of $\alpha$ helix and $\beta$ sheet at far UV molar ellipticity readings with decreased of $\alpha$ helix from 37% (TLN) to 20.6% (TLN-PBZ) and then to 19.8% (TLN-PBZ-Mg). Meanwhile, a further decrease of $\beta$ sheet from 32.6% (TLN) to 18.7% (TLN-PBZ) and then to 11.0% (TLN-PBZ-Mg) was also observed.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia
sebagai memenuhi keperluan ijazah Master Sains

**SINTESIS DAN PENCIRIAN METALLOTHERMOLYSIN SEMISINTETIK**

Oleh

**SYARAJATUL ERMA KHALID**

**April 2008**

**Pengerusi : Profesor Madya Mohd. Basyaruddin Abdul Rahman, PhD**

**Fakulti : Sains**

Modifikasi enzim telah melalui pelbagai strategi dan mengalami evolusi tersendiri. Thermolysin (TLN) merupakan sejenis enzim yang telah diekstrak dari *Bacillus thermoproteolyticus rokko*. Ia telah diubahsuai menjadi metalloenzim semisintetik yang terdiri daripada gabungan enzim, ligan dan ion logam. Beberapa ligan yang digunakan adalah benzamidina (BEN), 1,10-phenantrolina (PHN), p-aminobenzamidina (PBZ) dan etanolamina (ETA). Ion-ion logam yang terlibat pula adalah magnesium ($Mg^{2+}$), zink ($Zn^{2+}$), kalsium ($Ca^{2+}$) dan nikel ($Ni^{2+}$). Aktiviti metallothermolysin semisintetik diperolehi dari tindak balas hidrolisis azocasein. Antara empat jenis ligan tersebut, kompleks TLN-PBZ menunjukkan aktiviti spesifik yang tertinggi (2219.5 U/mg) pada kepekatan optimum PBZ, 0.6 mM. Ujikaji diteruskan dengan penambahan $Mg^{2+}$ pada kompleks TLN-PBZ yang menghasilkan aktiviti spesifik tertinggi berbanding ion logam lain (39406.4 U/mg). Kepekatan optimum $Mg^{2+}$ adalah 0.08 mM. Beberapa parameter telah diuji

kesan pH, suhu, masa tindak balas dan kestabilan suhunya. Sebagai keputusannya, metallothermolysin semisintetik ini dapat mengekalkan persekitaran dalam larutan penimbal tris-HCl pada pH 7.0 manakala tindak balas optimumnya adalah pada 80$^{o}$C selama 3 jam (peratusan aktiviti relatif sebanyak 96.7 %). Metallothermolysin semisintetik ini stabil suhu walaupun melalui pra pemanasan pada suhu 80$^{o}$C di mana ia mampu bertindak balas dengan kadar 90 %.

Analisis spektroskopi seperti Ultra lembayung boleh nampak (UV/Vis), Ultra lembayung fluoresen (UV/*Fluorescence*) spektrofotometer dan *Circular Dichroism* (CD) spektropolarimeter digunakan bagi tujuan pencirian aset optikal metalloenzim semisintetik. Keputusan UV/Vis menunjukkan berlaku anjakan pada bacaan panjang gelombang apabila perlekatan PBZ ke TLN berlaku ($\lambda_{max}$ dari 279 ke 274 nm) dan bersifat hipokromik ($\lambda_{max}$ dari 274 nm ke 272 nm) apabila $Mg^{2+}$ ditambah. Perubahan bacaan UV/Vis disokong keputusan bacaan UV/*Fluorescence* yang menunjukkan perubahan bacaan panjang gelombang bagi spektrum TLN-PBZ (373.2 nm) dan anjakan spektrum terus berlaku (374.0 nm) bagi TLN-PBZ-Mg. Keputusan CD spektropolarimetri mengusulkan berlakunya perubahan struktur protein α heliks dan β *sheet* di mana bacaan unit molar eliptisiti α heliks menurun dari 37.0 % (TLN), ke 20.6 % (TLN-PBZ) dan seterusnya 19.8 % (TLN-PBZ-Mg). Bagi struktur β *sheet* penurunan dari 32.6 % (TLN) kepada 18.7 % (TLN-PBZ) sehingga ke 11.0 % (TLN-PBZ-Mg).

## ACKNOWLEDGEMENTS

*Alhamdulillah*, praises to Allah s.w.t. for giving me the strength to endure all problems and complete this study.

I wish to express my sincere appreciation and gratitude to my supervisor, Associate Professor Dr. Mohd Basyaruddin Abdul Rahman for his patience and persistent encouragements. Thank you to my supervisory committee, Professor Dr. Mahiran Basri and Professor Dr. Abu Bakar Salleh for their great concern, advices and invaluable assistance from the beginning till the end of this study.

Thank you also to the staff members of the Department of Chemistry who were so helpful and cooperative in many ways during the course of the study.

I wish to thank all my friends and members of Lab 401, Kak Salina, Kak Yati, Ita, Redzuan, Pei Sin, Lam, Us, Mona, Azizah, Nora, Shie Ling, Hasmah and Casey for the friendship and for making my stay in UPM a memorable one with many sweet memories and experiences. Thank you for being friends in need.

My deep expression was also extended to Biochemistry lab mates especially, Kak Ina, Kak Ain, Aiman, Ghani, Leow, Kok Whye, Ropandi and the others for their valuable help.

Not forgotten my colleague in Mardi, Ezy, Dayana, Nisa, Sabeetha and Kak Ju, thank you for your help and support.

Finally, my deepest appreciation goes to my parents, papa and mama, for their never-ending moral and constant support during my studies. Not forgetting, my special thanks to my husband, Wan Ahmad Marzuki B. Wan Ahmad for his patience and to my son, Wan Muhammad Syamim, you are my inspiration.

This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the degree of Master of Science. The members of the Supervisory Committee were as follows:

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Date: 10 July 2007

## DECLARATION

I declare that the thesis is my original work except for quotations and citations which have been duly acknowledged. I also declare that is has not been previously, and is not concurrently submitted for any other degree at UPM or at any other institution.

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**SYARAJATUL ERMA BINTI KHALID**

Date: 24 June 2008

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## LIST OF ABBREVIATIONS

**Enzyme**

| | |
|---|---|
| Protease X from *Bacillus Thermoproteolyticus rokko* (Thermolysin enzyme) | TLN |

**Ligands**

| | |
|---|---|
| p-Aminobenzamidine | PBZ |
| Benzamidine | BEN |
| Ethanolamine | ETA |
| 1, 10- phenanthroline | PHN |

**Metals/ Metal ions**

| | |
|---|---|
| Cadmium | Cd |
| Calcium ion (II) | $Ca^{2+}$ |
| Cromium | Cr |
| Cuprum | Cu |
| Ferum | Fe |
| Kalium | K |
| Lithium | Li |
| Magnesium ion (II) | $Mg^{2+}$ |
| Manganese | Mn |
| Natrium | Na |
| Nickel ion (II) | $Ni^{2+}$ |
| Zinc ion (II) | $Zn^{2+}$ |

**Amino Acids**

| | |
|---|---|
| Alanine | Ala |
| Asparagine | Asn |
| Aspartate | Asp |
| Glutamine | Glu |
| Glysine | Gly |
| Histidine | His |
| Isoluecine | Ile |
| Leucine | Leu |
| Lysine | Lys |
| Methionine | Met |
| Serine | Ser |
| Tyrosine | Tyr |
| Valine | Val |
| Adenosine triphosphate | ATP |

**Spectrocopy Instruments**

| | |
|---|---|
| Circular Dichroism | CD |
| Ultra-violet/ Fluoresence | UV/Fluo |
| Ultra-violet/Visible | UV/Vis |

**Units**

| | |
|---|---|
| Absorbance | [A] |
| Centimeter | cm |
| Dalton | Da |
| Gram | g |
| Kilo-dalton | kDa |
| Liter | L |
| Microgram | μg |
| Microliter | μl |
| Mililiter | ml |
| Miligram | mg |
| Molar | M |
| Molar absorptivity | ε |
| Molar ellipticity | θ |
| Optical Density | OD |
| Temperature | $^{o}C$ |
| Unit | U |
| Wavelength | nm |

I certify that an Examination Committee has met on 11 April 2008 to conduct the final examination of name of Syarajatul Erma Binti Khalid on her Master of Science thesis entitled “Synthesis and Characterization of Semisynthetic Metallothermolysin” in accordance with Universiti Pertanian Malaysia (Higher Degree) Act 1980 and Universiti Pertanian Malaysia (Higher Degree) Regulations 1981. The Committee recommends that the student be awarded the Master of science.

Members of the Examination Committee were as follows:

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Date: 26 June 2008

# CHAPTER 1

## INTRODUCTION

Enzymes as biocatalyst are in high demand by the industrial field for their high rates and high reaction specificity and stereoselectivity. Enzymes can accomplish the reactions at mild pH, temperatures and pressures, thereby it consumes less energy. As enzymes are non-toxic, it minimizes problem of downstream waste and by-product disposal in ecologically acceptable processes.

Native enzyme nowadays has gone through evolution where many modifications have been done to fulfill these demands (Abdul Rahman, 1993). One of the modification types is by synthesizing a semisynthetic enzyme. Semisynthetic enzyme refers to an artificial enzyme that was developed at a define site with cofactor or new functional group for its novel properties and unique features. It consists of enzyme, ligand and metal (Rawling and Barrett, 1995).

In this regards, isolated thermolysin from *Bacillus thermoproteolyticus rokko* was selected to be modified to produce a new enzyme known as the semisynthetic metalloenzyme. Thermolysin is known for its high thermostability and many studies have been done by researchers from all over the world (Boonyaras *et al.*, 2000).

According to Ory *et al*., (1998), an enzyme has regions on its surface where small molecule or ion can bind. Some binding sites on the surface of enzymes may allow binding but only to a limited range of the chemical compounds. The design for development of semisynthetic enzyme was based on the use of protein pockets that can accommodate ligand as an intermediate between the pockets at the surface of the enzyme and metal. According to Conn *et al*., (1987), approximately one-third of known enzymes has metals as part of their structure, which requires metals to be added for activity or is further activated by metals. Several additions of ligand and metal were highlighted in this study to observe the best complex synthesized that was capable in enhancing or inhibiting the enzyme reaction.

Recently, protein engineering and chemical modification has become a successful valuable tool for creating or improving protein function for practical uses. Therefore introducing cofactors or other reactive moieties into proteins provides enormous flexibility for the design of semisynthetic catalysts that could be employed for a variety of purposes especially to enhance its reaction activity (Distefano and Davies, 1997).

Understanding the structural and functional significance of these ligand and metal effects requires a specialized array of sophisticated instrumentation and techniques as well as the expertise to use them. It is only through a detailed understanding of structure and function that enzymes can be selected or redesigned to perform industrially relevant catalysis (Kazlauskas, 2000). Owing to its inherent sensitivity, simplicity and to some extent

selectivity, UV/Visible (UV/Vis) and UV/Fluoresence spectroscopy were among the selected spectroscopy techniques for more valuable structural proposal (Donald *et al*., 2001). Circular Dichroism (CD) spectropolarimetry was also used for prediction of the secondary structure of a protein that was modified.

This research focused on developing positive biocatalysts for a variety of purposes especially in pharmaceutical and chemical industries. Therefore the objectives of this study are:

1) To design and synthesize metalloenzyme as biocatalyst in bio-based industries.

2) To study the characterization and optimization of the modified enzyme.

3) To evaluate the activity of the novel semisynthetic metallothermolysin through hydrolysis of azocasein.

4) To analyze the semisynthetic metalloenzyme structure using modern spectroscopy.

In order to fulfill the above objectives, the native thermolysin had gone through screening before modification. The proteolytic activity was determined by using azocasein as the substrates. Identified as hydrolases

enzyme which cleave peptide bond, thermolysin catalyzed amide (peptide) bond hydrolysis in protein or peptide substrates.

Parameters involved were the optimum pH, temperature, reaction time and thermostability. These screening results helped us to determine the suitable environment of the native enzyme. Modification steps of the native enzyme were held by mixing the enzyme with ligands and metal ions (one at a time) to form a couple of protein complexes. Then these protein complexes were purified and assayed. The total activity of each complex had been compared among the protein complexes and native enzyme activity. The protein complex that yields highest total activity was chosen for further investigation. The chosen complex was characterized and optimized for its pH surrounding, temperature and thermostability.

This was followed by confirming its structure with an electronic probe such as UV/Vis, UV/Fluorescence spectroscopy and CD spectropolarimeter. The use of UV/Vis proved to us on how different molecules absorbed spectrum showed a number of absorption bands corresponding to structural groups within the molecule at different wavelengths. The same absorbance understanding goes to UV/Fluorescence spectroscopy that had been used and applied to study the fundamental physical processes of molecules and one of them was in structure–function relationships and interactions of biomolecules such as proteins and nucleic acids. CD spectropolarimeter on the other hand will predict protein secondary structure by obtaining information from the UV region of the spectrum.