
**UNIVERSITI PUTRA MALAYSIA**

***HETEROLOGOUS EXPRESSION AND CHARACTERIZATION OF L-ASPARAGINASE FROM MARINE Photobacterium sp. STRAIN J15***

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**FBSB 2014 21**

# HETEROLOGOUS EXPRESSION AND CHARACTERIZATION OF L-ASPARAGINASE FROM MARINE *Photobacterium* sp. STRAIN J15

By

**MOHD ADILIN BIN YAACOB**



**Thesis submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfillment of the requirements for the degree of Master of Science**

**January 2014**

## DEDICATION

I dedicate my dissertation work to my family and many friends. A special feeling of gratitude to my loving father and sisters, Yaacob, Rosliza, Roslina,Roslima,Rosliah, Amir Hakimi and Nur Farah Dilyani whose words of encouragement and push for tenacity ring in my ears. I also dedicate this dissertation to my many friends and supervisor who have supported me throughout the process. I will always appreciate all they have done, especially Zulhilmi for helping me develope my technology skills, and Dr. Adam Leow Thean Chor for the many hours of proofreading. I dedicate this work and give special thanks to my lovely wife Atiqah and my wonderful daughter Aamilah Hannani for being there for me throughout the entire master program. Both of you have been my best cheerleaders.

Abstract of 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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**January 2014**

**Chairman: Adam Leow Thean Chor, PhD**

**Faculty: Biotechnology and Biomolecular Sciences**

L-asparaginase catalyzes the conversion of the L-asparagine to L-aspartate and ammonia. Although L-asparaginase is one of the main components used in chemotherapy, toxicity problem associated with glutaminase activity of commercial asparaginase products such as Elspar, oncaspar and Erwinaze derived from *Escherichia coli* and *Erwinia crysanthemi* becomes a major limitation in cancer treatment. The aim of the current study is to isolate L-asparaginase (J15 asparaginase) gene from *Photobacterium sp.* strain J15 and characterize J15 asparaginase biochemically by molecular expression and *in silico* modeling. Genome mining revealed an open reading frame of 1011 bp coding for J15 asparaginase gene from halo-tolerant *Photobacterium sp.* strain J15. The J15 asparaginase gene was isolated by using specific primer flanking the full length of gene overexpressed in pET-32b vector and transformed into *E. coli* strain Rosetta-gami B (DE3) pLysS. The J15 asparaginase was purified to homogeneity by using two-step chromatographic: $Ni^{2+}$-Sepharose affinity chromatography and Q-Sepharose anion exchange chromatography. The final specific activity and yield of the enzyme achieved from these steps were 20 U/mg and 49.2 %, respectively.

The functional dimeric form of J15-asparaginase with molecular weight of ~70 kDa was characterized. The optimum temperature and pH was at 25 °C and pH 7 respectively. It was stable in the presence of 1 mM $Ni^{2+}$ and $Mg^{2+}$, but was inhibited by $Mn^{2+}$, $Fe^{3+}$ and $Zn^{2+}$. J15 asparaginase actively hydrolyzed its natural substrate L-asparagine, but had low activity towards L-glutamine. The $T_m$ for J15 asparaginase was about 51 °C, as revealed by denatured protein analysis of Circular Dichorism (CD) spectra. The $K_m$, $K_{cat}$, $K_{cat}/K_m$ of J15 asparaginase was 0.76 mM, 3.2 $S^{-1}$, and 4.21 $S^{-1}mM^{-1}$, respectively. Structural analysis of J15 asparaginase was analysed by homology modeling and Molecular Dynamic (MD) simulations. The J15 asparaginase model was validated by using Ramachandran plot, and ERRAT 2.0. The monomer of J15 asparaginase comprises of 31 % α-helixs and 18.3 % ß-sheet. RMSD value of 0.436 with 76.85 % identities was recorded when the J15 asparaginase superimposed with template (pdb: 2OCD). MD simulation for 10 ns

was carried out to examine conformational changes of J15-asparaginase structure at different temperatures (25 °C, 45 °C, and 65 °C) and it was found that at 25 °C, J15 asparaginase was most stable hence able to hydrolyze the substrate at optimum rate. The function of mobile lid-loop at the active site was analyzed by using MD simulation at 10 ns. $Tyr_{24}$, $His_{22}$, $Gly_{23}$, $Val_{25}$, and $Pro_{26}$ residues might directly involve in 'open' and 'closed' of lid-loop conformation to facilitate the conversion of substrate during enzymatic reaction. Low glutaminase activity possessed by J15 asparaginase might serve as an ideal candidate to be used as enzyme-drug in cancer treatment.

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

**PENGEKSPRESAN SECARA HETEROLOGI DAN PENCIRIAN L-ASPARAGINASE DARIPADA MARIN *Photobacteria* sp. STRAIN J15**

Oleh

**MOHD ADILIN BIN YAACOB**

**Januari 2014**

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L-asparaginase memangkinkan penukaran L-asparagine kepada L-aspartate dan ammonia. Walaupun L-asparaginase adalah salah satu komponen utama yang digunakan dalam kemoterapi, masalah keracunan yang berkaitan dengan aktiviti glutaminase daripada asparaginase komersial seperti Elspar, oncaspar dan Erwinaze diperolehi daripada *Escherichia coli* dan *Erwinia crysanthemi* menjadi had utama dalam rawatan kanser. Tujuan kajian semasa adalah untuk mengasingkan L-asparaginase (J15 asparaginase) gen daripada *Photobacterium sp.* J15 dan mencirikan J15 asparaginase secara biokimia dengan menggunakan kaedah biologi molekul dan model komputer. Perlombongan genom mendedahkan L-asparaginase jenis I (J15 asparaginase) dengan panjang gen 1011 bp dari spesies *Photobacteria* strain J15. Enzim ini telah dihasilkan dalam kuantiti yang banyak menggunakan vektor pET-32b dan *E. coli* Rosetta- gami B (DE3) pLysS sebagai hos serta ditulenkan menggunakan dua langkah kromatografi: affiniti Nikel Sepharose dan Q-Sepharose. Aktiviti spesifik dan hasil yang dicapai daripada kaedah penulenan ini adalah masing-masing sebanyak 20 U/mg dan 49.2 %.

J15 asparaginase berbentuk dimerik dengan anggaran saiz sebesar ~70 kDa diperolehi dengan suhu optimum dan pH masing-masing pada 25 °C dan pH 7. J15 asparaginase stabil dengan kehadiran 1 mM $Ni^{2+}$ dan $Mg^{2+}$, tetapi direncatkan oleh $Mn^{2+}$, $Fe^{3+}$, dan $Zn^{2+}$. J15 asparaginase aktif mengkatalisis substrat semulajadi L-asparagine tetapi mempunyai aktiviti yang rendah kepada L-glutamine. Analisis menggunakan CD spektrum menunjukan $T_m$ untuk J15 asparaginase adalah kira-kira 51 °C. Nilai $K_m$, $K_{cat}$, $K_{cat}$ / $K_m$ dari J15 asparaginase masing-masing adalah 0.76 mM, 3.2 $S^{-1}$, dan 4.21 $S^{-1}mM^{-1}$. Struktur J15 asparaginase telah dianalisis dengan model homologi dan simulasi Molekul Dinamik (MD). Model J15 asparaginase telah disahkan dengan menggunakan plot Ramachandran dan ERRAT 2.0. Monomer J15 asparaginase terdiri daripada 31% α-helik dan 18.3% ß-sheets. Nilai RMSD sebanyak 0.436 dengan 76.85 persamaan identiti dicatatkan apabila J15 asparaginase dibanding

dengan struktur acuan (pdb: 2OCD). Simulasi MD selama 10 ns telah dijalankan untuk mengkaji perubahan struktur J15 asparaginase pada suhu yang berbeza (25 °C, 45 °C dan 65 °C) dan ia menunjukan bahawa J15 asparaginase paling stabil pada 25 °C dan mampu menkatilisis substrat pada tahap optimum. Fungsi gelung mudah alih di tapak aktif juga dianalisis dengan menggunakan simulasi MD pada 10 ns. $Tyr_{24}$, $His_{22}$, $Gly_{23}$, $Val_{25}$, dan $Pro_{26}$ mungkin terlibat secara langsung dalam membuka dan menutup gelung mudah alih untuk memudahkan penukaran substrat semasa tindak balas enzim. J15 asparaginase yang menunjukan glutaminase aktiviti yang rendah merupakan calon yang ideal untuk digunakan dalam rawatan kanser

**ACKNOWLEDGEMENT**

First and foremost, I would like to thank to my supervisor of this project Dr. Adam Leow Thean Chor for valuable guidance, and advice. He inspired me greatly to work in this project and his willingness to motivate me contributed tremendously to my project.A special gratitude I give to my research group (EMTECH group) especially Prof. Raja Noor Zaliha Raja Abd Rahman, Prof Dato' Abu Bakar Salleh, and Dr. Mohd Shukuri Bin Mohamad Ali, without whose knowledge and assistance this study would not have been successful. Furthermore, I also would like to acknowledge with much appreciation my lab mates especially Saif, Zarir, Azmir, Rahim, Opah, Zulhilmi, Hapiz, Hisham, Ropandi, kak Yati, Kak Fiza, Menega, Dura, Ira, Muadz, Kak Atie, Maya, Randa, Elly, Kak Ana, Kak Dayana, Naim and other EMTECH member whose contribution in stimulating suggestion and encouragement helped me to coordinate my project throughout these years (2010-2013)

I especially thank my father who always love and care about me. I love him so much and I would not have made this far without him. The best outcome from all these years is finding my best friends, soul mate, and wife. I married the best person for me. Wan Atiqah Najiah binti Wan Hasan is the only who can appreciate my quirkiness and sense of humor. She has been a true and great companion and has unconditionally loved me during my good and bad times.

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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## DECLARATION

**Declaration by the student**

I hereby confirm that:

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| | |
|---|---|
| µg/mL | Microgram per millilitre |
| µM | Micromolar |
| Å | Amstrong |
| $Å^2$ | Amstrong square |
| C | Celsius |
| cm | Centimeter |
| dH2O | Distilled water |
| DNA | Deoxyribonucleic acid |
| g | Gram |
| g/L | Gram per Litre |
| h | Hour |
| IPTG | Isopropyl β-D Thiogalactoside |
| kbp | Kilobasepair |
| kDa | KiloDalton |
| MD | Molecular Dynamic |
| mg | Milligram |
| mL | Milliliter |
| mM | Millimolar |
| Nm | Nanometer |
| OD | Optical density |
| PCR | Polymerase chain reaction |
| pI | Isoelectrical point |

| | |
|---|---|
| RMSD | Root Mean Square Deviation |
| SASA | Solvent Accessible Surface Area |
| SDS | Sodium dodecyl sulphate |
| SDS-PAGE | Sodium dodecyl sulphate polyacrylamide gel electrophoresis |
| *sp.* | Species |
| U/mg | Unit per milligram |
| U/mL | Unit per millilitre |
| w/v | Weight per volume |
| YASARA | Yet Another Scientific Artificial Reality Application |
| µg | Microgram |
| µl | Microliter |

# CHAPTER 1

## INTRODUCTION

Amino acid degrading enzyme L-asparaginase (EC 3.5.1.1) catalyzed the conversion of L-asparagine into aspartic acid and ammonia. The enzyme has been used for 40 years as chemotherapeutic agent to treat cancer cell especially Acute Lymphoblastic Leukemia (ALL) (Verma *et al.,* 2007) by degrading the L-asparagine in blood serum. Reduction of L-asparagine in circulatory system will selectively kill the tumor cell without affecting normal cells (Kotzia and Labrou, 2007). L-asparaginase has been used as diagnostic biosensor as the amount of ammonia produced during enzymatic reaction is directly correlates to the level of L-asparagine in a patient blood. Other than that, L-asparaginase also has successively been used in food industry. The pretreatment of carbohydrate rich food with L-asparaginase before baking has been proven to reduce the formation of acrylamide.

L-asparaginase is ubiquitous in environment but the enzymes from bacteria have obtained considerable interest because bacteria can be easily grown to high cell density thus economically viable. To date, L-asparaginase has been characterized from various bacterial genera. Despite of numerous studies on Gram positive bacteria such as *Pseudomonas fluorescence*, *Seratia marcencens*, *Mycobacterium sp.* and *Staphylococcus sp.,* their intrinsic glutaminase activity which could result serious toxicity restrict their clinical application. However, L-asparaginase from Gram negative bacteria such as *Erwinia crysanthemi* (ErA) and *Escherichia coli* (ECII) were found to be the most effective, owing to less immunorelated toxicity (Vidya *et al.,* 2011). Their application however has limitation that restricted their application as effective cancer treatment. Because ECII is a bacterial protein, hypersensitivity ranging from mild allergic reaction to death is often observed during treatment (Moola *et al.,* 1990). The development of neutralizing antibodies after a few series of treatment also reduces the efficacy of ECII. In such cases, ErA is often used as substitute since it has different pharmacokinetic and immunogenic properties. However, ErA has shorter half-life than ECII and need to administer frequently. Other than that, the used of ErA also associated with neurotoxicity which partially attributed to its glutaminase activity (Narta *et al.,* 2007)

Asparaginases from different environment have different biochemical and kinetic properties hence exploring a new source of L-asparaginase with different serological properties but same therapeutic effect is crucial to produce safer enzyme-drug based in cancer treatment (Eden *et al.,* 1990). The enzyme from marine microbe is an interesting subject to be further investigated because they have diverse range of enzymatic activity and capable of catalyzing various biochemical reaction. Marine enzyme biotechnology can offer novel biocatalysts with properties like high salt tolerance, hyperthermostability, barophilicity, cold adaptivity, and ease in large-scale cultivation (Debahish *et al.,* 2005). Halophilic bacteria possess many hydrolytic enzymes and capable of functioning under condition that lead to precipitation and denaturation of most protein. In addition of that, human body contain the element of salt such as sodium, potassium and calcium in almost the same proportion of ocean and this could provide microbial product such as enzyme that could be safer, more

effective with no or less toxicity when used for therapeutic application in human being.

In the present study, the genome from marine Gram negative *Photobacterium sp.* strain J15 was isolated, partially sequenced and annotated. The gene sequence coded for L-asparaginase was isolated and characterized. In order to have better understanding on this new isolated gene, the following strategic were implemented:

1) To isolate J15 asparaginase gene from *Photobacterium sp.* strains J15 and express in *E.coli* expression system.
2) To purify and characterize J15 asparaginase biochemically
3) To predict J15 asparaginase structure by using Homology modelling and Molecular Dynamic simulation.

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