UNIVERSITI PUTRA MALAYSIA

CLONING, EXPRESSION, AND PURIFICATION OF RIBOFLAVIN SYNTHASE FROM PHOTOBACTERIUM SP. J15

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CLONING, EXPRESSION, AND PURIFICATION OF RIBOFLAVIN SYNTHASE FROM *PHOTOBACTERIUM* SP. J15

By

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A Special Dedication to

My Father for his unconditional love and support; for his believing in me and to lig
to find the best way.

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

CLONING, EXPRESSION, AND PURIFICATION OF RIBOFLAVIN SYNTHASE FROM *PHOTOBACTERIUM* SP. J15

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Chair: Mohd Shukuri Bin Mohamad Ali, PhD
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Riboflavin synthase (RiS) is a homotrimeric enzyme that catalyzes the final reaction of riboflavin (vitamin B_2) biosynthesis from two molecules of 6,7-dimethyl-8-ribityllumazine. Gram-negative bacteria and certain yeasts are unable to absorb riboflavin or riboflavin derivatives from the environment. The RiS is nonexistent in humans while it only presents in bacteria and yeasts, which are dependent on their own production of riboflavin and will not survive in lack of the RiS function; hence the RiS is a potential target for the development of antimicrobial agents to target Gram-negative pathogens. The aim of this research was to clone and express the RiS in heterologous system and investigate the oligomerization of the RiS monomers. The RiS in current study is from a Malaysian isolated strain, *Photobacterium* sp. strain J15. Protein sequence alignment of this RiS shows high similarity to the RiS of *E. coli*. Analysis of the DNA sequence of this riboflavin synthase shows 6 rare codons, in which common *E. coli* strains are not able to supply the corresponding tRNAs, thus the *E. coli strain* Rosetta-gami B (DE3) pLysS was selected as the expression host. The gene encoding RiS was cloned into pET-32b(+) vector, which carried ampicillin resistance gene and utilized T7 promoter for high-level expression of a *Trx*-His tagged protein. The heterologous expression of RiS was performed by transformation of recombinant pET-32b(+) vector into *E. coli* Rosetta-gami B(DE3)pLysS. High-level expression of soluble RiS was optimized for expression temperature at 20 °C, inducer (IPTG) concentration at 1 mM, and time of induction for 20 h. The purification of the His-tagged enzyme was performed by affinity chromatography and was subjected to western-blot for verification. The purified fusion enzyme was cleaved by thrombin to remove Trx-His tag and purified by anion exchange chromatography to get the mature enzyme. Specific activity of mature RiS was significantly higher than fusion RiS. The size of mature RiS was estimated as 26.5 kDa by gel filtration chromatography. Site directed mutagenesis of I190V and chimerization of RiS was experimented to improve the trimer formation of quaternary structure of RiS but it didn not affect the trimerization of monomers, therefore, formation of trimer of RiS monomers was confirmed by cross-linking experiments. A melting point of 42.3 °C and a secondary structure composition of 13.6 % helix, 25.1 % beta, 12.5 % turn and 38.5 % random was measured by Circular-dichroism (CD) analysis. Through this work the RiS of *Photobacterium* sp. J15 was successfully cloned and expressed into heterologous system. The RiS is

found to function only in dimeric or trimeric form. The availability of functional enzyme by heterologous expression enables further characterization and structural studies towards drug development at pharmaceutical level.

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

PENGKLONAN, EKSPRESI, DAN PENULENAN RIBOFLAVIN SINTASE DARI *PHOTOBACTERIUM* SP. J15

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Riboflavin sintase (RiS) merupakan enzim homotrimeric yang memangkin tindak balas terakhir dalam biosintesis riboflavin (vitamin B2) daripada dua molekul 6,7-dimetil-8-ribityllumazine. Bakteria Gram-negatif dan yis tidak mampu menyerap riboflavin atau hasilan riboflavin dari alam sekitar. RiS ini juga tidak wujud pada manusia dan ia hanya ditemui dalam bakteria dan yis, yang bergantung kepada pengeluaran riboflavin mereka sendiri dan tidak akan dapat hidup jika kekurangan fungsi RiS; oleh itu RiS menjadi sasaran untuk pembangunan agen antimikrob terutamanya patogen Gram-negatif. Tujuan kajian ini adalah untuk mengklon dan mengekspresi RiS dalam sistem heterologous dan akhirnya untuk menyiasat oligomerisasi RiS dari monomernya. RiS dalam kajian semasa ini adalah berasal dari strain terpencil di Malaysia; *Photobacterium* sp. J15. Penjajaran jujukan protin Ris ini menunjukkan persamaan yang tinggi kepada RiS dari *E. coli*. Analisis jujukan DNA daripada riboflavin sintase ini menunjukkan 6 codon yang jarang ditemui, di mana strain *E. coli* biasa tidak mampu untuk membekalkan tRNAs yang diperlukan, dengan itu strain *E. coli* Rosetta-Gami B (DE3) pLysS dipilih untuk menjadi perumah ekspresi. Gen yang mengekod RiS telah diklon ke dalam vektor pET-32b (+), yang membawa gen rintangan terhadap ampicillin dan menggunakan penganjur T7 untuk mengekspresi protein yang telah ditanda dengan Trx-His pada tahap tinggi. Ekspresi RiS secara heterologous dilakukan dengan menjalankan transformasi rekombinan pET-32b (+) ke dalam vektor *E. coli* Rosetta-Gami B (DE3) pLysS. Ekspresi RiS yang larut pada tahap tinggi telah dioptimumkan suhu eskpresinya pada 20 ° C, kepekatan pencetus (IPTG) sebanyak 1 mM, dan masa induksi selama 20 jam. Penulenan enzim yang ditanda dengan His telah dilakukan dengan kromatografi kecenderungan GDQ ³ZHVWHUQ EORW´ WHODK GLODNXNDQ XQWXN SHQJHVDKDQ (Q gabungan yang tulen telah dileraikan oleh thrombin untuk membuang penanda Trx-His dan ditulenkan pula dengan kromatografi pertukaran anion untuk mendapatkan enzim yang matang. Aktiviti spesifik untuk RiS matang adalah jauh lebih tinggi daripada RiS yang bergabung. Saiz RiS yang matang dianggarkan sebanyak 26.5 kDa oleh kromatografi penurasan gel. Mutagenesis tapak yang diarah bagi I190V dan chimerisasi RiS telah dieksperimenkan untuk meningkatkan pembentukan trimer bagi struktur kuaterner RiS tetapi ia tidak menjejaskan trimerisasi daripada monomer, oleh itu pembentukan trimer daripada monomer RiS telah disahkan oleh eksperimen silang-hubung. Takat lebur pada 42.3 °C dan komposisi struktur

sekunder sebanyak 13.6% heliks, 25.1% beta, 12.5% lengkuk dan 38.5% rawak, diukur dengan analisis circular-dichroism (CD). RiS daripada *Photobacterium* sp. J15 telah berjaya diklon dan diekspres di dalam sistem heterologous. RiS daripada *Photobacterium* sp. J15 didapati hanya akan berfungsi dalam bentuk dimeric atau trimeric. Kehadiran enzim yang berfungsi daripada ekspresi heterologous membolehkan pencirian lanjut dan kajian struktur dijalankan.

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TABLE OF CONTENTS

	Page
ABSTRACT	iii
ABSTRAK	v
ACKNOWLEDGEMENTS	vii
APPROVAL	viii
DECLARATION	x
LIST OF TABLES	xv
LIST OF FIGURES	xvi
LIST OF ABBREVIATIONS	xviii

CHAPTER

1	**INTRODUCTION**		1
	1.1	Problem statement	2
	1.2	Objectives	2
2	**LITERATURE REVIEW**		3
	2.1	Riboflavin	3
	2.2	Riboflavin Synthase	3
		2.2.1 Application of riboflavin synthase	5
		2.2.2 Heterologous expression of riboflavin synthase	5
	2.3	Multimeric enzymes	7
	2.4	Mechanism and structure of riboflavin synthase	7
	2.5	Site directed mutagenesis	13
	2.6	Protein engineering and chimeragenesis	14
	2.7	Cross-linking of protein-protein interactions	14
	2.8	Sources of riboflavin synthase	14
		2.8.1 *Photobacterium*	15
3	**MATERIALS AND METHODS**		16
	3.1	Materials	16
	3.2	General methods	16
	3.3	Strains and plasmids	16
	3.4	Cloning of RiS gene using pTrcHis TOPO TA Expression Kit	17
		3.4.1 Extraction of genomic DNA of *Photobacteium* sp. strain J15	17
		3.4.2 Amplification of riboflavin synthase gene	17
		3.4.3 Cloning of RiS gene into pTrcHis vector	18
		3.4.4 Preparation of *E. coli* competent cells	18
		3.4.5 Transformation of Recombinant pTrcHis into *E. coli* Top10	18
		3.4.6 Direct PCR analysis of positive clones	18

3.4.7 Preparation of glycerol stock from positively transformed colony 19
3.5 Expression of RiS gene in *E. coli* Top10 19
3.6 SDS-PAGE analysis of riboflavin synthase expression in *E. coli* Top10 19
3.7 Cloning of RiS gene into pET-32b(+) Vector 20
3.7.1 Amplification of RS gene 20
3.7.2 Double Digestion of PCR product and Vector 20
3.7.3 Purification of digested RiS gene and pET-32b(+) vector 21
3.7.4 Ligation of RiS gene into pET32b(+) Vector 21
3.7.5 Heat-Shock transformation of *E. coli* Top10 21
3.7.6 Colony PCR 21
3.7.7 Sequencing of recombinant plasmid (pET/RiS) 21
3.8 Expression of RiS gene in *E. coli* Rosetta-gami B(DE3)pLysS 21
3.8.1 Extraction of recombinant PET-32b(+) plasmid 21
3.8.2 Transformation of recombinant plasmid pET/RiS into *E. coli* Rosetta-gami B(DE3)pLysS 22
3.9 Expression of RiS in *E. coli* Rosetta-gami B(DE3)pLysS 22
3.9.1 Optimization of inducer concentration effect on RiS expression 22
3.9.2 Optimization of Temperature effect on RiS Expression 22
3.9.3 Optimization of incubation time of RiS expression 22
3.10 Purification of recombinant riboflavin synthase 23
3.10.1 Preparation of crude lysate 23
3.10.2 Affinity purification of RiS 23
3.11 Western-Blot analysis of purified riboflavin synthase 23
3.12 Tag cleavage and purification of mature riboflavin synthase 23
3.12.1 Cleavage of fusion RiS by thrombin 23
3.12.2 Desalting of thrombin cleavage mixture 24
3.12.3 Anion-exchange chromatography purification of mature RiS 24
3.13 Assay of riboflavin synthase 24
3.14 Gel filtration and size determination of mature RiS 24
3.15 Circular-Dichroism (CD) analysis of mature RiS 25
3.16 Protein modeling and sequence analysis of RiS 25
3.16.1 Multiple sequence alignment of riboflavin synthase 25
3.16.2 Protein structure analysis 25
3.17 Protein engineering for assembly of quaternary structure 25
3.17.1 Site Directed Mutagenesis (SDM) 25
3.17.1.1 Transformation of mutant plasmid pET/I190V into *E. coli* Rosetta-gami B(DE3)pLysS 26
3.17.1.2 Expression of the mutant RiS 26
3.17.1.3 Analysis of trimeric formation of RiS 26
3.17.2 Chimerization of RiS by replacing the C-terminal helix 26
3.17.3 Cross-linking of RiS monomers 27

4 RESULTS AND DISCUSSION 28
4.1 Cloning of RiS Gene in pTrcHis vector 28
4.1.1 Genomic DNA extraction 28

4.1.2 PCR cloning of RiS gene 29
4.1.3 Analysis of positive transformants 30
4.2 Expression of RiS gene in *E. coli* Top10 32
4.2.1 SDS-PAGE analysis of RiS expression in *E. coli* Top10 32
4.3 Examining the codon usage of RiS Gene 33
4.4 Restriction site analysis of RiS gene and Primer design 34
4.5 Cloning of RiS gene in pET-32b(+) system 35
4.5.1 RiS Gene amplification by RiS primers 35
4.5.2 Double digestion and ligation of RiS gene and vector 35
4.5.3 Transformation of recombinant plasmid pET/RiS into *E. coli* Top10 36
4.5.4 Analysis of positive transformants 36
4.6 Transformation of recombinant pET/RiS into *E. coli* Rosetta-gami B(DE3)pLysS 38
4.6.1 Expression of RiS in *E. coli* Rosetta-gami B(DE3)pLysS 39
4.6.2 SDS-PAGE analysis of expressed proteins 40
4.7 Optimization of temperature effect on RiS expression 41
4.8 Optimization of IPTG concentration on RiS expression 41
4.9 Optimization of incubation time on RiS expression 45
4.10 Purification of His-tagged RiS 46
4.11 Western blot analysis of purified RiS 47
4.12 Activity assay evaluation of RS enzyme 48
4.13 Thrombin cleavage of fusion RiS 49
4.14 Size determination of mature RiS by gel filtration 51
4.15 Circular Dichroism analysis of mature RiS 52
4.16 Sequence analysis and Protein Modelling of RiS 53
4.17 Site directed mutation of RiS 57
4.18 Chimerization of RiS from *Photobacterium* sp. J15 60
4.19 Cross-linking of RiS from *Photobacterium* sp. J15 61

5 CONCLUSION AND RECOMMENDATION FOR FUTURE RESEARCH
5.1 General discussion 63
5.2 Conclusion 63
5.2 Recommendation for future research 64

REFERENCES 65
APPENDICES 70
BIODATA OF STUDENT 77
LIST OF PUBLICATIONS 78