UNIVERSITI PUTRA MALAYSIA

EXPRESSION, CHARACTERIZATION, AND RATIONAL DESIGN OF CHALCONE SYNTHASE FROM *Physcomitrella patens*

IFFAH IZZATI BINTI ZAKARIA

FBSB 2012 45

EXPRESSION, CHARACTERIZATION, AND RATIONAL DESIGN OF CHALCONE SYNTHASE FROM *Physcomitrella patens*

By

IFFAH IZZATI BINTI ZAKARIA

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirements for the Degree of Doctor of Philosophy

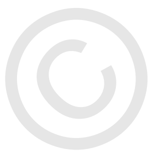
November 2013

© COPYRIGHT UPM

COPYRIGHT

All material contained within the thesis, including without limitation text, logos, icons, photographs and all other artwork, is copyright material of Universiti Putra Malaysia unless otherwise stated. Use may be made of any material contained within the thesis for non-commercial purposes form the copyright holder. Commercial use of material may only be made with the express, prior, written permission of Universiti Putra Malaysia.

Copyright © Universiti Putra Malaysia



Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfillment of the requirement for the degree of Doctor of Philosophy

EXPRESSION, CHARACTERIZATION, AND RATIONAL DESIGN OF CHALCONE SYNTHASE FROM *Physcomitrella patens*

By

IFFAH IZZATI BINTI ZAKARIA

November 2013

Chairman : Prof Raja Noor Zaliha Raja Abd. Rahman, PhD
Faculty : Biotechnology and Biomolecular Science

Flavanoids are plant secondary metabolites synthesized by polyketide synthase (PKS) in plants, fungi and bacteria. The members of the chalcone synthase (CHS) superfamily, also known as the type III PKSs, function as the key entry enzyme of the flavanoid biosynthesis. Flavanoids are found in liverworts and mosses (bryophyte) that are thought to be the land plants ancestor. Although hundred of CHSs genes from various plant species have been successfully clone, expressed and studied, limited information is available on the CHS from the bryophytes. The model moss, *Physcomitrella patens* is currently the only bryophyte whose genome has been sequenced and its genome contains at least 17 putative type III PKS genes. Among them, a CHS gene was shown to be basal to all plant type III PKS genes in phylogenetic trees. *P. patens* CHS exhibited similar kinetic properties and substrate preference profiles as those of higher plants. This suggest that the *P. Patens* CHS may exhibit similar mechanism as the other land plants CHSs. Due to the unavailability of the *P. Patens* CHS crystal structure, the main aim of this work is to gain some insight on the structure and mechanism of the *P. Patens* CHS.

Apart form the unavailability of *P. patens* CHS crystal structure, the major bottleneck of this work is to obtain the *P. patens* CHS due to the slow plant growth rate and low (less than 1%) extraction yield. Therefore, to speed up the process of obtaining the enzyme, the *P. patens* CHS has been successfully cloned and expressed in *E. coli* strain BL21 (*DE3*) plysS. However, the formation of inclusion bodies has become a major disadvantage of this approach. As alternative, *P. patens* CHS was secreted into the medium using a bacteriocin release protein expression vector. Secretion of *P. patens* CHS into the culture media was achieved by co-expression with a psW1 plasmid encoding bacteriocin release protein in *E. coli* Tuner (*DE3*) plysS.

© COPYRIGHT UPM

The optimized conditions were incubation of cells for 20 hours with 40 ng/ml mitomycin C at OD_{600} induction time of 0.5 was found to be the best condition for chalcone synthase secretion. The recombinant *P. patens* CHS was purified to 1.78-fold with 88.1% yield and specific activity of 4.26 U/mg by affinity chromatography technique using Ni^{2+} Sepharose Fast Flow resin. The enzyme optimum pH and temperature were 7.0 and 30 °C, respectively. In addition, the enzyme was found to be stable up to 50 °C. Several crystallization attempts of *P. patens* CHS was carried out using vapour diffusion techniques, however, X-ray data processing was unable to be performed due to the weak diffraction spots obtained from the *P. Patens* crystals. Weak diffraction spots obtained might be due to poor crystal packing, the presence of impurities, nonspecific aggregations and crystal damaged by X-ray beam.

Consequently, to study the structure and mechanism of *P. patens* CHS, combination of homology modeling and site-directed mutagenesis was conducted. The *P. patens* CHS structure was built using the *M. sativa* CHS crystal structure as a template. Based on the overall assessment, the quality of the modeled structure was comparable to template. Through the modeled *Pp*CHS structure, showed the same four catalytic residues of the active site conserved in other CHS superfamily. Due to the function of the catalytic Cys170 in the first binding (initiation reaction) of the starter molecule on the catalytic active site, it was targeted as a critical residue. To investigate the effect of Cys170 substitutions towards the *P. patens* enzyme activity, two mutants were constructed, C170 R and C170S.

From the mutant's data analysis, it could be concluded that the catalytic residue Cys170 plays an important role in the binding of substrate onto the active site. Substitution of Cys170 has lead to several changes in its interaction among the other residues resides in the active site cavity. The changes affect the binding capacity, cavity volume of the active site and the overall protein structure volume which decreased the production of reaction products. Apart, the mutant's physical properties such as temperature, pH and stability were found to be affected as well.

© COPYRIGHT UPM

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk Ijazah Doktor Falsafah

PENZAHIRAN, PENCIRIAN, DAN REKA BENTUK RASIONAL *Physcomitrella patens* CHALCONE SYNTHASE

Oleh

IFFAH IZZATI BINTI ZAKARIA

November 2013

Pengerusi : Prof. Raja Noor Zaliha Raja Abd. Rahman , PhD
Fakulti : Bioteknologi dan Sains Biomolekul

Flavanoid adalah kompoun yang dihasilkan oleh polyketide synthase (PKS) daripada tumbuhan, kulat, dan bakteria. Kumpulan chalcone synthase yang juga tergolong didalam kumpulan III PKSs, berperanan sebagai entri utama laluan metabolik flavanoid. Flavanoid didapati didalam lumut dan kulut (bryophyta) yang dikenalpasti berasal dari keturunan yang sama dengan tumbuhan daratan. Walaupun ratusan gen CHS daripada pelbagai spesis tumbuhan telah berjaya diklon, diekspres dan dikaji, kurang maklumat mengenai CHS daripada kumpulan bryophta. *Physcomitrella patens* merupakan satu-satunya kumpulan bryophyta yang seluruh jujukan genom telah berjaya dirungkaikan yang merangkumi 17 kumpulan III PKS gen. *P. patens* CHS menunjukkan ciri-ciri kinetik dan pemilihan substrak yang same seperti tumbuhan daratan yang lain. Ini menunjukkan bahawa *P. patens* CHS mempunyai mekanisma yang sama seperti CHS tumbuhan daratan yang lain.

Selain daripada ketiadaan struktur kristal *P. patens* CHS, cabaran yang sebenar ialah untuk mendapatkan enzum *P. patens* CHS disebabkan oleh kadar tumbesaran tumbuhan yang sangat perlahan dan hasil pengekstrakan yang rendah (kurang dari 1%). Oleh itu, untuk mempercepatkan proses mendapatkan enzim, *P. patens* CHS telah berjaya diekspreskan dalam sistem *E. coli* BL21(*DE3*) plysS. Namun begitu, kebanyakan protein yang diekspres adalah dalam bentuk protein tidak larut. Untuk mengatasi masalah ini, *Pp*CHS diekspres terus ke media melalui 'bacteriocin release protein (BRP)' teknik. Rembesan *Pp*CHS terus ke media dicapai melalui penggunaan psW1 vektor dalam system *E. coli* Tuner (DE3) plysS.

Rembesan enzim chalcone synthase terus ke media adalah optimum di bawah beberapa parameter iaitu 20 jam inkubasi dengan 40 ng/ml mitomycin C pada OD_{600nm} masa induksi 0.5. Seterusnya, rekombinan *P. patens* CHS berjaya ditulenkan kepada 1.78 kali ganda dengan hasil 88.1% serta mempunyai aktiviti spesifik sebanyak 4.26 U/mg

© COPYRIGHT UPM

dengan menggunakan kaedah kromatografi afiniti. Enzim ini mempunyai pH and suhu optima masing masing pada 7.0 dan 30 °C. Enzim ini didapati stabil sehingga 50 °C. Beberapa percubaan kristalografi *P. patens* CHS dilakukan menerusi kaedah resapan wap, namun begitu, pemprosesan awal data X-ray bagi *P. patens* CHS yang dihasilkan gagal diperolehi. Pemprosesan data X-ray tidak berjaya diperolehi mungkin disebabkan oleh beberapa factor seperti penyusunan molekul kristal yang tidak teratur, kehadiran pelbagai bendasing, pengagregatan protein atau protein kristal rosak semasa dibelau oleh sinaran X-ray.

Oleh itu, untuk mengkaji struktur dan mekanisma *P. patens* CHS, gabungan teknik homology modelling dan mutasi telah digunakan. Struktur model *P. patens* CHS dibina menggunakan kristal struktur *M. sativa* CHS sebagai templat. Berdasarkan penilaian-penilaian ini, struktur ramalan ini mempunyai tahap persamaan yang tinggi dengan templatnya iaitu struktur kristal *Ms*CHS, seterusnya dapat diterima dan digunakan untuk kajian seterusnya. Menerusi struktur model *Pp*CHS, didapati 4 residu yang sama sperti CHS dari kumpulan tumbuhan yang lain. Disebabkan oleh fungsi residu Cys170 sebagai tempat mengikat substrat didalam rongga aktif, ia dianggap sebagai residu yang kritikal. Oleh itu, untuk mengkaji kesan penggantian residu Cys170 terhadap aktivit *Pp*CHS, dua mutant telah dihasilkan iaitu mutant C170R dan mutant C170S.

Berdasarkan data-data analisis kedua-dua mutant, didapati bahawa residu Cys170 memainkan peranan yang penting dalam proses mengikat substrat pada rongga aktif *Pp*CHS. Penggantian residu C170S telah menyebabkan perubahan interaksi diantara residu-residu didalam rongga aktif. Perubahan ini memberi kesan pada kapasiti mengikat, size rongga aktif dan size keseluruhan protein menyebabkan penghasilan produk berkurangan. Selain itu, sciri-ciri fizikal mutant turat berubah seperti suhu, ph dan kestabilan protein.

© COPYRIGHT UPM

ACKNOWLEDGEMENTS

To the long bumpy ride taken, thanks Allah The Almighty for the experience, knowledge and wisdom obtained. Thank you for the blessings.

For showing me the true meaning of passion, commitment and self-motivation, my deepest gratitude and appreciation to my supervisor Prof. Dr. Raja Noor Zaliha Raja Abd Rahman and the other committee co-supervisors. Thank you for being such an inspiration and to all the guidance given.

Not forgetting to all my labmates, which includes each and everyone in Lab 140. The friendship and bond built between us make this years of journey as another exciting chapter of mine. Thank you for your sincere help, kindness and to the memories we shared.

To the unconditionally love, support, and trust, I owe it to both of my parents, Mdm. Norhuda Ridwan and Mr. Zakaria Hashim. Thank you for always having faith in me despite whatever happens. Also to all family members, it wouldn't be the same without you.

Lastly, to my heart and soul, Mr. Azmeer Ariff and little one Aryan Ariff, it has been a tough battle, but you both make it seems worthwhile. Thank you for just being here.

© COPYRIGHT UPM

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

Raja Noor Zaliha Raja Abd Rahman, D. Eng
Professor
Faculty Biotechnology and Biomolecular Science
Universiti Putra Malaysia
(Chairman)

Abu Bakar Salleh, PhD
Professor
Faculty Biotechnology and Biomolecular Science
Universiti Putra Malaysia
(Member)

Mahiran Basri, PhD
Professor
Faculty of Science
Universiti Putra Malaysia
(Member)

BUJANG BIN KIM HUAT, PhD
Professor and Dean
School of Graduate Studies
Universiti Putra Malaysia

Date:

© COPYRIGHT UPM

DECLARATION

Declaration by Iffah Izzati Binti Zakaria

I hereby confirm that:

- this thesis is my original work;
- quotations, illustrations and citations have been duly referenced;
- this thesis has not been submitted previously or concurrently for any other degree at any other institutions;
- intellectual property from the thesis and copyright of thesis are fully-owned by Universiti Putra Malaysia, as according to the Universiti Putra Malaysia (Research) Rules 2012;
- written permission must be obtained from supervisor and the office of Deputy Vice-Chancellor (Research and Innovation) before thesis is published (in the form of written, printed or in electronic form) including books, journals, modules, proceedings, popular writings, seminar papers, manuscripts, posters, reports, lecture notes, learning modules or any other materials as stated in the Universiti Putra Malaysia (Research) Rules 2012;
- there is no plagiarism or data falsification/fabrication in the thesis, and scholarly integrity is upheld as according to the Universiti Putra Malaysia (Graduate Studies) Rules 2003 (Revision 2012-2013) and the Universiti Putra Malaysia (Research) Rules 2012. The thesis has undergone plagiarism detection software.

Signature: ________________________ Date: ___________________

Name and Matric No.: ____

© COPYRIGHT UPM

Declaration by Members of Supervisory Committee

This is to confirm that:

- the research conducted and the writing of this thesis was under our supervision;
- supervision responsibilities as stated in the Universiti Putra Malaysia (Graduate Studies) Rules 2003 (Revision 2012-2013) are adhered to.

Signature: ____________________
Name of
Chairman of
Supervisory
Committee: ____________________

Signature: ____________________
Name of
Member of
Supervisory
Committee: ____________________

Signature: ____________________
Name of
Member of
Supervisory
Committee: ____________________

Signature: ____________________
Name of
Member of
Supervisory
Committee: ____________________

© COPYRIGHT UPM

TABLE OF CONTENTS

	Page
ABSTRACT	i
ABSTRAK	iii
ACKNOWLEDGEMENTS	v
APPROVAL	vi
DECLARATION	vii
LIST OF TABLES	xiii
LIST OF FIGURES	xiv
LIST OF ABBREVIATIONS	xvii

CHAPTER

1	**INTRODUCTION**	1
2	**LITERATURE REVIEW**	
	2.1 Flavanoids	
	2.1.1 Applications of flavonoids	3
	2.1.2 Metabolic engineering of flavonoids	5
	2.2 Chalcone synthase	
	2.2.1 Chalcone synthase properties	6
	2.2.2 Chalcone synthase structure and function	7
	2.2.3 Chalcone synthase reaction mechanism	10
	2.3 Expression of chalcone synthase	12
	2.4 Purification of chalcone synthase	13
	2.5 Site-directed mutagenesis of chalcone synthase	
	2.5.1 The role of Cys-His dyad	14
	2.6 *Physcomitrella patens*	15
	2.7 Secretory expression of recombinant protein	16
	2.7.1 Bacteriocin release protein	17
	2.8 Crystallization	
	2.8.1 Factors influencing protein crystallization	22
	2.8.2 Additives	23
	2.8.3 Crystallization of chalcone synthase	25
	2.9 X-ray diffraction	
	2.9.1 Dynamic light scattering	26
	2.9.1.1 The application of dynamic light scattering	27
3	**MATERIALS AND METHODS**	
	Preparation of media and solution	28

© COPYRIGHT UPM

3.1 Cloning of the full-length *Pp*CHS gene

3.1.1 Plasmid extraction of pCAMBIA-*Pp*CHS 28
3.1.2 Amplification of *Pp*CHS gene 29
3.1.3 Purification of PCR product 29
3.1.4 Preparation of competent cells 30
3.1.5 Cloning of PCR product 30
3.1.6 Screening for positive clones 30
3.1.7 Sequencing for positive clones 31
3.1.8 Transformation of recombinant pET32(a)/*Pp*CHS 31
3.2 Intracellular expression of *Pp*CHS

3.2.1 Detection of recombinant *Pp*CHS by SDS-PAGE 32
3.2.2 Detection of recombinant *Pp*CHS by Western-blot 32
3.3 Enzyme assay of chalcone synthase 33
3.4 Extracellular expression of recombinant *Pp*CHS

3.4.1 Co-transformation of psW1 into pET32(a)/*Pp*CHS 33
3.4.2 Screening for positive clones 34
3.5 Quasi-lysis studies 34
3.6 Optimization of extracellular expression of *Pp*CHS 34
3.6.1 Effect of inducer concentration on *Pp*CHS secretion 34
3.6.2 *Pp*CHS secretion at different induction A_{600nm} 35
3.6.3 Effect of temperature on *Pp*CHS secretion
35

3.6.4 Time-course analysis of *Pp*CHS secretion 35

3.7 Analysis of *Pp*CHS secretion by SDS-PAGE analysis 35
3.8 Purification of recombinant *Pp*CHS 35
3.9 Characterization of purified *Pp*CHS 36
3.9.1 The effect of pHs on *Pp*CHS activity 36
3.9.2 The effect of temperature on *Pp*CHS activity 36
3.9.3 Thermal stability study on *Pp*CHS activity 36
3.10 Crystallization of *Pp*CHS

3.10.1 Thrombin cleavage treatment 37
3.10.2 Ion exchange chromatography 37
3.10.3 Crystallization screening of *Pp*CHS crystal

3.10.3.1 Precipitating reagents 38
3.10.3.2 Protein and precipitant concentration 38
3.11 X-ray diffraction

3.11.1 Preparation of cryoprotectant 38
3.11.2 In-house data collection 39
3.12 Dynamic light scattering (DLS) analysis 39
3.13 Structure prediction of *Pp*CHS 39

© COPYRIGHT UPM

3.14 Site-directed mutagenesis *Pp*CHS

3.14.1 Construction of mutants 39
3.14.2 Preparation of *Pp*CHS mutants 40
3.14.3 Screening for positive mutants 41
3.15 Protein expression in *E. coli* and enzyme extracts 41
3.16 Purification of C170R mutant and C170S mutant 41
3.17 *Pp*CHS mutant's activity determination and characterization

3.17.1 The effect of temperature on *Pp*CHS mutant's activity 42
3.17.2 Thermal stability studies on *Pp*CHS mutant's activity 42
3.17.3 The effect of pH on *Pp*CHS mutant's activity 42
3.17.4 Half-life studies on the *Pp*CHS mutants 42
3.18 Statistical Analysis 42

4 **RESULTS AND DISCUSSION**
4.1 Cloning of the full length *Pp*CHS cDNA 43

4.2 Expression of the recombinant pET32(a)/*Pp*CHS 50
4.3 Determination of *Pp*CHS product 52
4.4 Extracellular expression of recombinant *Pp*CHS 58
4.5 Optimization of *Pp*CHS secretion

4.5.1 Effect of inducer concentration on *Pp*CHS secretion 61
4.5.2 Effect of cell density (A_{600nm}) on *Pp*CHS secretion 63
4.5.3 Effect of induction time on *Pp*CHS secretion 65
4.6 Purification of recombinant *Pp*CHS 66
4.7 Characterization of recombinant *Pp*CHS

4.7.1 The effect of pH on *Pp*CHS activity 69
4.7.2 The effect of temperature on *Pp*CHS activity 71

4.7.3 Thermal stability study of *Pp*CHS activity 73
4.8 Crystallization of *Pp*CHS

4.8.1 Purification of *Pp*CHS for crystallization 73
4.8.2 Crystallization screening 78
4.8.3 Optimization of *Pp*CHS crystallization 82
4.8.4 Pre-diffraction *Pp*CHS crystal 84
4.8.5 Hydrodynamic properties as a function of cysteine 85
4.9 Structure prediction of *Pp*CHS 87
4.10 Evaluation of predicted 3D structure 89
4.11 Mutations by site directed mutagenesis 92

© COPYRIGHT UPM

4.12 Preparation of purified C170R and C170S mutant 94
4.13 The *Pp*CHS mutant's analysis 99
4.14 Characterization of recombinant *Pp*CHS, C170R, and C170S mutants

4.14.1 The effect of temperature on the *Pp*CHS mutants 101
4.14.2 The effect of pH on *Pp*CHS mutants 103
4.14.3 Half-life studies of *Pp*CHS mutants 105
4.14.4 Comparisons of the recombinant *Pp*CHS, C170R, and C170S mutant 108

5 CONCLUSION AND RECOMMENDATION
5.1 Conclusion 110
5.2 Recommendation 111

REFERENCES 112
APPENDICES 128
BIODATA OF STUDENT 144
LIST OF PUBLICATIONS 145

© COPYRIGHT UPM