MOLECULAR AND FUNCTIONAL INDICATION OF *CHALCONE SYNTHASE* IN *BOESENBERGIA ROTUNDA*

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THESIS SUBMITTED IN FULFILLMENT OF THE
REQUIREMENTS FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

INSTITUTE OF BIOLOGICAL SCIENCES
FACULTY OF SCIENCE
UNIVERSITY OF MALAYA

KUALA LUMPUR

2014

UNIVERSITY MALAYA

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ABSTRACT

Chalcone synthase (CHS) is a key enzyme in flavonoids biosynthesis pathway, which catalyzes the condensation of three acetate residues from malonyl-CoA with p-coumaroyl-CoA to form naringenin chalcone. This step is the first committed step of the phenylpropanoid pathway, which regulates other sub-branches to produce flavonoids, isoflavonoids, anthocyanin, chalcone, and other flavonoids compounds in plants. Several studies have shown that the flavonoids and chalcones are pharmaceutically active in *Boesenbergia rotunda*, but the purification is often impossible due to the low concentration of these flavonoids. Most studies recently focused on the individual genes of the pathway such as *CHS* gene to overproduce certain compounds like panduratin A. Comparison of *CHS* gene sequence from different species revealed that *CHS* gene is structurally conserved. In this study, a core fragment of *CHS* gene was amplified using nested PCR. The amplicon of 584bp length encoding ~194 amino acids was confirmed as part of the second exon of *CHS* gene, however the complete triad active site was not present in the core fragment of *B. rotunda* CHS protein. The core fragment showed that the nucleotide and amino acid sequence of the second exon of *CHS* gene is variable. Gene expression analysis indicated the presence of *CHS* transcript in leaves, rhizomes, roots, and shoot base of *B. rotunda* with the highest expression level in shoot base. The full-length *B. rotunda CHS* gene was then amplified and cloned from *B. rotunda* rhizome using rapid amplification of cDNA ends. The amplicon of 1,257bp length containing a coding sequence of 1,176bp, which codes for 391 amino acids with the molecular mass of 43.22kDa and a predicted isoelectric point of 6.79 was obtained. Comparative and bioinformatic analyses revealed that the deduced protein of all nine variants (HQ176338-HQ176346) of *B. rotunda* CHS protein were highly homologous to CHSs from other plant species. Phylogenetic analysis indicated that the *B. rotunda* CHS protein was in a subgroup with *Dendrobium* CHS. The prediction of the secondary

structure of all nine variants of *B. rotunda* CHS protein mainly showed α-helix and extended strand. The prediction of three-dimensional structure of nine variants of *B. rotunda* CHS protein showed the highest similarity to alfalfa CHS (1CGZ) having CHS-specific conserve motifs and the CHS-family signature sequence GFGPG. The docking analysis showed that panduratin A could not be the direct product of *B. rotunda* CHS protein.

ABSTRAK

Chalcone synthase (CHS) adalah enzim utama dalam biosintesis flavonoid, yang menjadi pemang pemeluwapan tiga sisa asetat dari malonyl-CoA dengan p-coumaroyl-CoA untuk membentuk naringenin Chalcone, yang merupakan langkah pertama yang diambil dalam laluan phenylpropanoid yang membawa kepada cawangan sampingan lain untuk menghasilkan flavonoid, isoflavonoids, anthocyanin, chalcone dan lain-lain dalam tumbuhan. Beberapa kajian telah menunjukkan sebatian *Boesenbergia rotunda*, seperti flavonoid dan chalcones adalah aktif dari segi farmaseutikal. Perbandingan rangkaian gen *CHS* dari pelbagai spesies menunjukkan bahawa gen *CHS* dipelihara dari segi strukturnya. Dalam kajian ini, serpihan teras DNA gen *CHS* telah dikukuhkan menggunakan kaedah PCR bersarang dan telah mengesahkan bahawa serpihan 584bp adalah milik exon kedua gen *CHS*, namun tapak kongsi gelap aktif enzim *CHS* tidak lengkap dalam protein sebahagian ~194 amino acids. Serpihan teras ini juga menunjukkan bahawa exon kedua gen *CHS* adalah sentiasa berbeza dalam rangkaian nukleotida dan asid amino. Berikutan serpihan teras itu, panjang DNA gen *CHS* (dilabelkan sebagai *B. rotunda CHS*) telah dikukuhkan dan diklon dari rizom Boesenbergia rotunda oleh pengukuhan pesat di hujung cDNA. Panjang cDNA B. rotunda *CHS* adalah 1257 bp dalam saiz yang mengandungi rangka bacaan terbuka (1,176 bp) mengekodkan 391 asid amino, massa molekul yang dikira 43.22kDa dan titik Isoelektrik yand diramal 6.79. Analisis perbandingan dan bioinformatic menunjukkan bahawa protein yang disimpulkan daripada *B. rotunda CHS* adalah sangat homolog kepada *CHS* daripada jenis tumbuhan yang lain. Protein *B. rotunda* CHS mempunyai CHS – motif dipelihara yang khusus dan CHS – tandatangan keluarga rangkaian GFGPG. Pemodelan molekul menunjukkan bahawa struktur sekunder *B. rotunda* CHS mengandungi terutamanya α-heliks dan lanjutan unting. Analisis filogenetik menunjukkan bahawa protein *B. rotunda* CHS adalah dalam kumpulan pengganti

bersamaan *CHS* daripada *Dendrobium*. Panjang rangkaian pengekodan gen *CHS* menunjukkan bahawa rangkaian nukleotida dan asid amino adalah berbeza-beza, di mana sembilan keupayaan *CHS* telah diperolehi dalam kajian ini. Untuk pengetahuan kita, ini adalah laporan pertama untuk menghuraikan pengasingan dan analisis molekul gen *CHS* dalam *B. rotunda*.

ACKNOWLEDGMENT

First and foremost I offer my sincere gratitude to God who supports me in every moments of my life as throughout my PhD program.

I would like to thank **Prof. Dr. Zulqarnain Mohamed** and **Prof. Dr. Rofina Yasmin Othman**, for all their support and knowledge, **Prof. Dr. Habibah Wahab** for her assistance and interest especially in the Bioinformatics part and **Prof. Dr. Norzulaani Khalid** for her guidance throughout the project.

I gratefully acknowledge Ministry of Science, Technology & Innovation, Malaysia, 53-02-03-1005, Institute of Research Management and Consultancy (IPPP), University of Malaya, Grant Number PS213-2008C, University of Malaya Fellowship Scheme, and Faculty of Science for the support and opportunity to participate in various workshops, trainings, and conferences.

My special appreciation goes to my lovely husband, Abod, my little angel, Elyaa, and my caring family who closely and patiently stand beside me and I am sure this could never be completed without their support.

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List of Abbreviation

3D	Three-dimensional
B. pandurata	*Boesenbergia pandurata*
B. rotunda	*Boesenbergia rotunda*
BAP	Benzylaminopurine
BLAST	Basic Local Alignment Search Tool
β-actin	Beta actin
β-ME	β-Mercaptoethanol
cDNA	Complementary Deoxyribonucleic Acid
CIA	Chloroform:isoamylalcohol
CHS	Chalcone Synthase
Ct	Threshold cycle
CTAB	Cetyltrimethylammonium Bromide
CTAB-NETS	Cetyltrimethylammonium Bromide-NaCl:EDTA:Tris:SDS
DEPC	Diethylpyrocarbonate
DNA	Deoxyribonucleic Acid
dNTP	Deoxyribonucleic Triphosphate
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
eEF1-α	Elongation Factor 1 alpha
EtBr	EthidiumBromide
GSPs	Gene Specific Primers
HCC	Hexamine Cobalt Chloride
IPTG	Isopropyl-β-D-thiogalactopyranoside

KCl	Potassium Chloride
KOAc	Potassium Acetate
LB	Lysogeny Broth
LiCl	Lithium Chloride
$MgCl_2$	Magnesium Chloride
$MgSO_4$	Magnesium Sulfate
mRNA	Messenger RNA
NAA	Napthtalene Acetic Acid
NaCl	Sodium Chloride
NaOAc	Sodium Acetate
NaOH	Sodium Hydroxide
NCBI	National Centre for Biotechnology Information
NETS	NaCl:EDTA:Tris:SDS
OD	Optical Density
Oligo dT	Oligo deoxytimidine
PCR	Polymerase Chain Reaction
PDB	Protein Data Bank
PEG	Polyethylene Glycol
pH	Potenz Hydrogen
PC	Phenol:Chloform
PCIA	Phenol:Chloroform:IsoamylAlcohol
Phyre	Protein Homology/analogY Recognition Engine
pI	Isoelectric Point
PKS	*Polyketide Synthase*
PVP	Polyvinylpyrrodine

RACE	Rapid Amplification cDNA Ends
$RbCl_2$	Rubidium Chloride
RNA	Ribonucleic Acid
rRNA	Ribosomal Ribonucleic Acid
RT-PCR	Reverse Transcription PCR
SDS	Sodium dodecyl sulfate
ssDNA	Single-strand DNA
TBE	Tris-Borate-EDTA
UBQ5	Ubiquitin 5
UTR	Untranslated Region
X-Gal	5-bromo-4-chloro-3-indolyl-beta-D-galacto-pyranoside

List of Symbols

Symbols	Description
bp	Base pairs
°C	Degree Celsius
%	Percentage
µl	Microliter
µg	Microgram
M	Molar
ml	Milliliter
mM	Millimolar
V	Voltage
A	Absorbance

CHAPTER 1

1.0 Introduction

1.1 Background

Chalcone synthase (*CHS*) (EC 2.3.1.74) is the type III of *polyketide synthase* (*PKS*) superfamily, which is well studied in plants. This gene catalyzes the first committed step in the flavonoids biosynthesis pathway also known as phenylpropanoids pathway. The flavonoids pathway produces many secondary metabolites that are directly involved in the interaction between plants and environment. The secondary metabolites include proanthocyanins, anthocyanins, phytoalexins, flavones, flavonoids, flavonols, and isoflavonoids.

Each intermediate in the flavonoids biosynthesis pathway possesses certain positive roles such as protection against UV and resistance against insects and pathogens; therefore, the products of the pathway empower the plants for better adaptation to the stressful environment. To determine the adaptive evolution of the flavonoids biosynthetic pathway, the study of the genes especially the well-studied *CHS* gene is significant.

CHS enzyme condenses three acetate units (C2) from malonyl-CoA molecule to a phenylpropanoid CoA such as 4-coumaroyl-CoA also known as p-coumaroyl-CoA. These two compounds are starter molecules of CHS enzyme. From the chemical point of view, this reaction is a Claisen-type and it is classified as a cyclisation reaction. The Claisen condensation is a carbon-carbon bond forming reaction that occurs between two esters e.g. malonyl-CoA and phenylpropanoid CoA. CHS enzyme catalyzes the formation of a naringenin chalcone also known as chalcone molecule, which is an

aromatic tetraketide and is the precursor of diverse flavonoids. CHS enzyme establishes the C15 skeleton of flavonoids compounds. One of the examples is the biosynthesis of anthocyanin in several plants.

From the genetic point of view, *CHS* gene is known as the representative member of *CHS* superfamily genes. These superfamily genes are similar in sequence, structure, and general catalytic principles. They are homodimers of 40-45kDa subunits containing about 389 amino acids, which contain a catalytic triad in the active site. This triad includes three amino acids of Cys, His, and Asn.

Molecular studies on the sequence of *CHS* gene have come to attention of researches in the recent years. The sequence of *CHS* gene in many plants from monocot, dicot, some gymnosperm species, and bacteria have been reported along with genetic engineering studies on the flavonoids pathway; however there is no report of molecular studies of *CHS* gene in *Boesenbergia rotunda* (*B. rotunda)*.

B. rotunda (L.) Mansf. Kulturpfl. is a common spice containing pharmaceutically active flavonoids compounds. As an example, chalcone and cardamonin exhibit appreciable anti-HIV protease inhibition (Cheenpracha et al., 2006). Flavanones, chalcones, cardamonin, and cyclohexenyl chalcone derivatives (CCDs) extracted from *B. rotunda* showed inhibition toward DEN-2 virus NS3 protease (Kiat et al., 2006b).

The two species of *Kaempferia pandurata* Roxb (*K. pandurata*) and *Boesenbergia pandurata* Holtt (*B. pandurata*) are closely related to *B. rotunda.* They are perennial herb and belong to the ginger (Zingiberaceae) family. These plants are cultivated in tropical countries such as Malaysia, Indonesia, and Thailand. Larsen et al. (1999) mentioned that both known species of *B. pandurata* and *B. rotunda* are the same species.

The *B. rotunda* rhizome has been commonly used as a condiment and an ingredient in Southern Asian food with a pungent taste. The rhizome has been used as a folk medicine to treat various diseases in the digestive system including dyspepsia, colic disorder, stomach discomfort, dysentery, and aphthous ulcer. It was used in the respiratory system including dry cough and dry mouth, in the reproduction system including leucorrhea, as an aphrodisiac to stimulate sexual desire, to remove general muscular pains, rheumatism, and fungal infection. In Thailand, for instance, the rhizome was used as self-medication by AIDS patients. The *B. pandurata* has been reported to exhibit antibacterial, antifungal, anti-inflammatory, analgesic, antipyretic, antispasmodic, antitumor, and insecticidal activities (Tewtrakul et al., 2003).

The root system of *B. rotunda* is an underground part consisting roots and rhizomes, while the shoot system is an aerial part where stems, leaves, and flowers grow. The roots have no vegetative buds, nodes or internodes. They are red or brown in color and are mainly involved in absorption of water and minerals from soil. Rhizomes are the underground-modified stems, which grow parallel to the earth surface. They have nodes and internodes as stem but have shoot buds (shoot base). They store food material and propagate vegetative parts. Studies on the extracted flavonoids compounds from *B. pandurata* rhizome revealed that four types of rhizome exist in this plant, which are different in color: yellow, black, red, and white. All of these rhizomes contain specific essential oils. For instance, studies reported the isolation of pinostrobin, alpinetin, boesenbergin A, boesenbergin B, panduratin A, methoxychalcone, cardamonin, and pinocembrin from yellow rhizome, whereas about eleven flavonoids from black rhizome; crotepoxide, zeylenol, boesenboxide, isopimaric, and methoxychalcone from white rhizome and panduratin A, hydroxypanduratin A, sakuranetin, pinostrobin, pinocembrin, dehydrokawain, boesenbergin A, rubranine from red rhizome.

Among the various flavonoids compounds in the flavonoids biosynthesis pathway, panduratin A isolated from *K. pandurata* possesses significant anti-inflammatory property in murine macrophages and induced ear edema in rats. It has antioxidative, cytotoxity, and cyclooxygenase (COX-2) inhibitory activity in mouse peritoneal macrophages and induces apoptosis in human colon cancer; therefore it is considered as an anti-tumorigenic compound. On human prostate cancer cells it showed anti-proliferative activity (Yun et al., 2006).

In *B. pandurata,* panduratin A exhibited strong antibacterial activity against *Porphyromonas gingivalis.* The compound showed anti-inflammatory activity by inhibiting the production of nitric oxide. Kiat et al. (2006a) showed that panduratin A and hydroxyl panduratin A have inhibitory activities toward DEN-2 NS2B/NS3 protease. In order to utilize the flavonoids compounds for pharmaceutical purposes, they can be extracted through chemical methods, but they are often produced at low levels in plants. Overproduction of these flavonoids compounds can also happen through molecular approaches, therefore the structure and function of the genes involved in the pathway should be studied.

Although there are many studies on significance of panduratin A, but the molecular pathway toward its production is still unknown in *B. rotunda*. This study aims to discover the structure and function of *CHS* gene in *B. rotunda* as the first step in the flavonoids biosynthesis pathway and whether panduratin A can be a potential direct product of CHS enzyme.

1.2 Research Problem Statement

The study of specific flavonoids such as panduratin A requires their purification but this is often impossible due to the low concentration of these flavonoids in the plants. The existence of similar flavonoids compounds in the plant makes it harder to extract and

purify the compound of interest. On the other hand, the production of the flavonoids compounds is restricted to the low growth rate of plants. Since the flavonoids compounds are secondary metabolites, they are produced at certain environmental conditions. The chemical and biological pathways are two possible ways to overproduce the flavonoids compounds. The chemical pathway that starts from a simple starting material requires extreme reaction conditions and toxic chemicals are involved, however the biological pathway focuses on the molecular biosynthesis of these compounds through the genes involved in the pathway.

Most of the studies on flavonoids pathway recently focus on the individual genes of the pathway to overproduce the specific compound. CHS is the central enzyme that controls the first committed step in the flavonoids biosynthesis pathway, therefore to overproduce a certain flavonoid compound like panduratin A in *B. rotunda*, the first step is to analyze the structure and function of *CHS* gene in this plant.

1.3 Aims and Objectives

This research aims to study the molecular and functional indication of *CHS* gene toward production of panduratin A in flavonoids biosynthesis pathway in *B. rotunda*. The objectives of the study are as follows:

1. To isolate and clone the complete sequence of *B. rotunda CHS* gene
2. To study the expression pattern of *CHS* gene in different tissues of *B. rotunda*
3. To predict the protein structure of CHS protein and dock the protein with panduratin A

1.4 Significance of the Study

The molecular and functional analyses of *CHS* gene helps to take the first step toward overproduction of certain flavonoids compounds like panduratin A in *B. rotunda*.

1.5 Research Flowchart

The research flowchart in Figure 1.1 shows the main stages of the study.