

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

FATEMEH SHAHHOSSEINI

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

ORIGINAL LITERARY WORK DECLARATION

Name of Candidate: Fatemeh Shahhosseini **I.C/Passport No:** K17154626

Registration/Matric No: SHC060033

Name of Degree: Doctor Of Philosophy

Title of Project Paper/ Research Report/ Dissertation/ Thesis (“This Work”):

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

Field of Study: Genetics and Molecular Biology

I do solemnly and sincerely declare that:

- (1) I am the sole author/writer of this Work;
- (2) This work is original;
- (3) Any use of any work in which copyright exists was done by way of fair dealing and for permitted purpose and any excerpt from, or reference to or reproduction of any copyright work has been disclose expressly and sufficiently and the title of the Work and its authorship have been acknowledge in this Work;
- (4) I do not have any actual knowledge nor do I ought reasonably to know that the making of this work constitutes an infringement of any copyright work;
- (5) I hereby assign all and every rights in the copyright to this work to the University of Malaya (UM), who henceforth shall be owner of the copyright in this work and that any written consent of UM having been first had and obtained;
- (6) I am fully aware that if in the course of making this work I have infringed any copyright whether intentionally or otherwise, I am be subject to legal action or any other action as may be determined by UM.

Candidate’s Signature

Date

Subscribed and solemnly declared before,

Witness’s Signature

Date

Name:

Designation:

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 yang 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 GFPGP. 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.

TABLE OF CONTENTS

ABSTRACT	II
ABSTRAK	IV
ACKNOWLEDGMENT	VI
LIST OF TABLES	XIV
LIST OF FIGURES.....	XVI
LIST OF ABBREVIATION	XXI
LIST OF SYMBOLS	XXIV
CHAPTER 1	1
1.0 Introduction	1
1.1 Background	1
1.2 Research Problem Statement.....	4
1.3 Aims and Objectives.....	5
1.4 Significance of the Study	5
1.5 Research Flowchart	6
CHAPTER 2	8
2.0 Literature Review	8
2.1 Secondary Metabolites	8
2.1.1 Flavonoids Compounds	9
2.1.2 Flavonoids Function	10
2.2 Flavonoids Biosynthesis Pathway.....	11
2.2.1 Chalcone	12
2.2.2 Panduratin A	13
2.3 Flavonoids Enzymes	15

2.3.1 Polyketide Synthases	15
2.3.2 Chalcone Synthase Multigene Family	17
2.3.2.1 Location of Chalcone Synthase Enzyme	19
2.3.2.2 Structure of Chalcone Synthase Enzyme.....	19
2.3.2.3 Reaction Mechanism of Chalcone Synthase Enzyme.....	22
2.3.2.4 Substrate Preferability of Chalcone Synthase Enzyme.....	24
2.3.2.5 Specificity of Chalcone Synthase Enzyme	25
2.4 Polyketide Synthase Gene Structure	26
2.4.1 Chalcone synthase Gene Structure	27
2.4.2 Chalcone Synthase Gene Location	31
2.4.3 <i>Chalcone Synthase</i> Gene Expression.....	31
2.4.4 Chalcone Synthase Gene Evolution.....	35
2.4.5 <i>Chalcone Synthase</i> Gene Duplication.....	36
2.4.6 <i>Chalcone Synthase</i> Phylogenetic Relationships	38
2.5 <i>Boesenbergia rotunda</i>	38
CHAPTER 3	41
3.0 Materials and Methods.....	41
3.1 Collection and Storage of Starting Material of <i>B. rotunda</i>	41
3.2 Preparation of Total DNA From <i>B. rotunda</i>	42
3.3 Determination of Yield and Purity of DNA.....	44
3.4 Obtaining Core Fragment of <i>B. rotunda CHS</i> Gene.....	45
3.4.1 Degenerate Primer Design	45
3.4.2 External Nested PCR and Internal Nested PCR	45
3.4.3 PCR Purification of Internal Nested PCR Product	46
3.5 Cloning Core Fragment of <i>B. rotunda CHS</i> Gene	48

3.5.1 Ligation Reaction Using pGEM®-T Easy Vectors and Rapid Ligation Buffer	50
3.5.2 Transformation of pGEM®-T Easy Vector Ligation Reaction	51
3.5.2.1 Competent Cell Preparation	52
3.5.2.2 Preparation of LB Agar and LB Broth	53
3.5.3 Blue/White Screening	53
3.5.4 Colony PCR	54
3.6 Plasmid Isolation	55
3.7 Restriction Enzyme Digestion	56
3.8 Preparation of Samples for Sequencing	57
3.8.1 Cycle Sequencing	57
3.8.2 Ethanol Precipitation	57
3.9 Expression of <i>B. rotunda</i> CHS Gene	58
3.9.1 Treatment of <i>B. rotunda</i> Callus	58
3.9.2 Optimization of Total RNA Extraction Methods	59
3.9.2.1 TRIzol® Method	59
3.9.2.2 Cetyltrimethylammonium Bromide-NETS Method	61
3.9.2.3 Cetyltrimethylammonium Bromide Method	62
3.9.2.4 RNA Isolation Kit	63
3.9.2.5 Gel Extraction Method	65
3.9.3 Determination of Yield and Purity of RNA	66
3.9.3.1 Agarose Gel Electrophoresis	66
3.9.3.2 Spectrophotometry	67
3.9.4 DNase Treatment of RNA Sample	67
3.9.5 Designing Gene Specific Primers	68
3.9.6 One-Step Reverse Transcription PCR	69

3.9.7 Real-Time Quantitative PCR Analysis	71
3.9.7.1 Synthesis of Single-Stranded DNA	71
3.9.7.2 Determination of Yield and Purity of Single-Stranded DNA	72
3.9.7.3 Selection of an Endogenous Control	73
3.9.7.4 Probe Selection	73
3.9.7.5 PCR Amplification of Single-Stranded DNA	74
3.10 Obtaining Full-length Coding Sequence of <i>B. rotunda</i> CHS Gene.....	75
3.10.1 5' End and 3' End Amplification of <i>B. rotunda</i> CHS Gene	75
3.10.1.1 5' RACE Amplification.....	76
3.10.1.2 3' RACE Amplification.....	77
3.10.2 QIAquick Gel Extraction of RACE Products	78
3.10.3 Designing Initiation-Termination Primers.....	80
3.10.4 Cloning of Full-length Coding Sequence of <i>B. rotunda</i> CHS Gene.....	81
3.10.5 Cloning of Full-length Sequence of <i>B. rotunda</i> CHS gene	82
3.11 Bioinformatics Studies.....	82
3.11.1 Homology Searching	82
3.11.2 Phylogenetic Tree Construction.....	83
3.11.3 Structure Prediction and Validation of <i>B. rotunda</i> CHS Protein.....	83
3.11.3.1 Secondary Structure	83
3.11.3.2 Three-Dimensional Structure.....	84
3.11.4 Characterization of Significant Amino Acids.....	84
3.11.5 Docking of <i>B. rotunda</i> CHS Protein and Panduratin A.....	84
3.11.6 Screening of <i>B. rotunda</i> CHS Variants Through Transcriptome Library....	85
CHAPTER 4	86
4.0 Results.....	86
4.1 Confirmation of CHS Gene Presence in <i>B. rotunda</i> Genome	86

4.1.1 Isolation of Core Fragment of <i>B. rotunda</i> <i>CHS</i> Gene Through Nested PCR	86
4.1.2 Purification of Nested PCR Product	88
4.1.3 Colony PCR of Core Fragment of <i>B. rotunda</i> <i>CHS</i> Gene	89
4.1.4 Sequence Analysis of Core Fragment of <i>B. rotunda</i> <i>CHS</i> Gene	91
4.2 Expression Studies of <i>B. rotunda</i> <i>CHS</i> Gene	96
4.2.1 Extraction of Total RNA from <i>B. rotunda</i> Tissues.....	96
4.2.2 Gene Specific Primers of <i>B. rotunda</i> <i>CHS</i> Gene	103
4.2.3 Reverse Transcription PCR of <i>B. rotunda</i> <i>CHS</i> Gene	106
4.2.4 Dissociation Curve Analysis of <i>B. rotunda</i> <i>CHS</i> Gene	107
4.2.5 Relative Quantification of <i>B. rotunda</i> <i>CHS</i> Transcript.....	109
4.3 Amplification of Full-length Sequence of <i>B. rotunda</i> <i>CHS</i> Gene.....	112
4.3.1 Preparation of RNA Sample From <i>B. rotunda</i> Rhizome	112
4.3.2 Cloning 5'RACE and 3'RACE Fragments.....	113
4.3.2.1 5'RACE Amplification Of <i>CHS</i> Gene	114
4.3.2.2 3'RACE Amplification of <i>CHS</i> Gene	116
4.3.3 Sequence Analysis of RACE Fragments of <i>B. rotunda</i> <i>CHS</i> Gene.....	119
4.3.4 Genomic PCR Amplification of Full-length Sequence of <i>B. rotunda</i> <i>CHS</i> Gene	123
4.3.5 Reverse Transcription-PCR Amplification of Full-length Coding Sequence of <i>B. rotunda</i> <i>CHS</i> Gene	125
4.3.6 Sequence Analysis of Full-length Gene and Full-length cDNA of <i>B. rotunda</i> <i>CHS</i>	126
4.4 Bioinformatics Analysis of <i>B. rotunda</i> <i>CHS</i> Gene.....	135
4.4.1 Comparative Studies of <i>B. rotunda</i> <i>CHS</i> Gene	135
4.4.2 Structure Prediction of <i>B. rotunda</i> <i>CHS</i> Protein	142
4.4.2.1 Primary Structure Alignment of <i>B. rotunda</i> <i>CHS</i> Variants.....	142

4.4.2.2 Secondary Structure Prediction of <i>B. rotunda</i> CHS Variants.....	145
4.4.2.3 Validation of Secondary Structure of <i>B. rotunda</i> CHS Variants.....	155
4.4.2.4 Prediction of Three-Dimensional Structure of <i>B. rotunda</i> CHS Variants	158
4.4.2.5 Superimpose of <i>B. rotunda</i> CHS Variants.....	165
4.4.2.6 Docking of <i>B. rotunda</i> CHS Protein With Naringenin.....	172
4.4.2.7 Screening of <i>B. rotunda</i> CHS Variants Through Transcriptome.....	175
CHAPTER 5	180
5.0 Discussion.....	180
5.1 <i>Boesenbergia rotunda</i>.....	180
5.2 Cloning and Characterization of Core Fragment of <i>B. rotunda</i> CHS Gene	181
5.3 RNA Extraction from <i>B. rotunda</i>	183
5.4 Gene Expression Analysis of <i>B. rotunda</i> CHS Gene	185
5.5 Cloning and Characterization of Full-Length <i>B. rotunda</i> CHS Gene.....	186
5.6 Sequence Variability of <i>B. rotunda</i> CHS Variants.....	189
5.7 Structure Prediction of <i>B. rotunda</i> CHS Protein	192
5.8 Substrate Preference of <i>B. rotunda</i> CHS Protein.....	194
CHAPTER 6	199
Conclusion	199
References.....	203
Appendixes.....	220
Appendix A: Expression Studies of <i>B. rotunda</i> CHS Gene	220
Appendix C: Sequence Alignment of RACE Fragments of <i>B. rotunda</i> CHS Gene	229
Appendix D: Sequence Alignment of Full-length <i>B. rotunda</i> CHS Gene.....	233

Appendix E: Full-length Sequence of Nine Variants of <i>B. rotunda</i> CHS Gene.	237
Appendix F: Bioinformatics Studies Of <i>B. rotunda</i> CHS Protein	246

List of Tables

Table 3-1 External and internal degenerate primers to perform nested PCR	45
Table 3-2 Sequence of GSPs ₁ and GSPs ₂ primers	68
Table 3-3 Sequence of endogenous controls primers	73
Table 3-4 Sequence of GSPs in Real-Time quantitative PCR	74
Table 3-5 Sequence of primers in RACE.....	75
Table 3-6 Sequence of GSPs ₃ and GSPs ₄ of <i>B. rotunda</i> CHS gene	80
Table 3-7 Sequence of Initiation-Termination primers.....	81
Table 4-1 Determination of DNA concentration of <i>B. rotunda</i> leaves through OD reading	87
Table 4-2 Determination of plasmid concentration through OD reading	91
Table 4-3 Determination of total RNA concentration through OD reading for <i>B. rotunda</i> tissues	103
Table 4-4 Nine variants of <i>B. rotunda</i> CHS gene with their accession numbers submitted to GenBank	135
Table 4-5 Identity score of nine variants of <i>B. rotunda</i> CHS protein with Chain A, CHS from alfalfa	145
Table 4-6 Identities of scope code of nine variants of <i>B. rotunda</i> CHS protein based on fold recognition.....	150
Table 4-7 High variability of four variants among nine variants of <i>B. rotunda</i> CHS protein.....	163
Table 4-8 Identities of Template PDB Code and Filtered Model of nine variants of <i>B. rotunda</i> CHS protein based on ModBase	165

Table 4-9 Isoelectric Point calculation for nine variants of <i>B. rotunda</i> CHS protein..	172
Table 4-10 Blast results of twenty-six unigenes of <i>B. rotunda</i> from treated callus.....	176
Table 4-11 Alignment of six unigenes with variant 8 of <i>B. rotunda</i> CHS gene	178
Table 4-12 Identity score of all nine variants of <i>B. rotunda</i> CHS gene with six unigenes.	179

List of Figures

Figure 1-1 Research flowchart.....	7
Figure 2-1 Phenylpropanoid metabolic pathway	12
Figure 2-2 Chemical structure of panduratin A	13
Figure 2-3 Three types of cyclization reaction catalyzed by plant type III PKSs	23
Figure 3-1 Four different tissues of <i>B. rotunda</i>	41
Figure 3-2 The promoter and multiple cloning sequence of pGEM®-T Easy Vectors.	48
Figure 3-3 pGEM®-T Easy Vector circle map.....	49
Figure 4-1 Extracted DNA samples of <i>B. rotunda</i> leaves on 0.8% agarose gel.....	86
Figure 4-2 Gel electrophoresis of gradient nested PCR of <i>B. rotunda</i> <i>CHS</i> gene.....	88
Figure 4-3 PCR purification of core fragment of <i>B. rotunda</i> <i>CHS</i> gene	89
Figure 4-4 Cloning steps of core fragment of <i>B. rotunda</i> <i>CHS</i> gene.....	90
Figure 4-5 Nucleotide and amino acid sequence of the core fragment of <i>B. rotunda</i> <i>CHS</i> gene.	92
Figure 4-6 The 150 Blast Hits of the core fragment sequence of <i>B. rotunda</i> <i>CHS</i> gene	94
Figure 4-7 Phylogenetic tree of the core fragment of <i>B. rotunda</i> <i>CHS</i> gene.....	95
Figure 4-8 Extracted total RNA from <i>B. rotunda</i> rhizome using TRIzol® method.....	97
Figure 4-9 Extracted total RNA from <i>B. rotunda</i> rhizome using RNA gel extraction method	98
Figure 4-10 Extracted total RNA from <i>B. rotunda</i> rhizome using CTAB method.....	99

Figure 4-11 Agarose Gel (1%) of extracted total RNA using modified CTAB method from four different tissues of <i>B. rotunda</i>	101
Figure 4-12 DNase treatment of total RNA samples of <i>B. rotunda</i>	102
Figure 4-13 Gradient PCR performed for two pairs of GSPs of <i>B. rotunda CHS</i> gene	104
Figure 4-14 Cloning steps of two specific fragments of <i>B. rotunda CHS</i> gene to confirm GSPs specificity	105
Figure 4-15 Nucleotide sequence of two specific fragments of <i>B. rotunda CHS</i> gene amplified using GSPs	106
Figure 4-16 Reverse transcriptase PCR for <i>B. rotunda CHS</i> gene	107
Figure 4-17 Dissociation curve analysis of <i>B. rotunda CHS</i> gene using SYBR® Green I dye.....	108
Figure 4-18 Dissociation curve analysis of actin gene using SYBR® Green I dye	109
Figure 4-19 Relative expression level of <i>CHS</i> gene in four different tissues of <i>B. rotunda</i>	110
Figure 4-20 Relative expression level of <i>CHS</i> gene in root, leaf, and rhizome of <i>B. rotunda</i>	111
Figure 4-21 Relative expression level of <i>CHS</i> gene in treated and untreated callus of <i>B. rotunda</i>	112
Figure 4-22 DNase treatment of RNA sample of <i>B. rotunda</i> rhizome for RACE analysis	113
Figure 4-23 Schematic structure of <i>B. rotunda CHS</i> gene.....	114
Figure 4-24 Cloning steps of 5'RACE of <i>B. rotunda CHS</i> gene with GSPs ₂ -R (340R)	115

Figure 4-25 Cloning steps of 5'RACE of <i>B. rotunda</i> <i>CHS</i> gene with GSP _{S1} -R (200R)	115
Figure 4-26 Cloning steps of 3'RACE of <i>B. rotunda</i> <i>CHS</i> gene with GSP _{S2} -F (480F)	117
Figure 4-27 Confirmation of specificity of 3'RACE fragment through PCR.....	118
Figure 4-28 Genomic PCR of <i>B. rotunda</i> <i>CHS</i> gene using GSP _{S3} and GSP _{S4}	119
Figure 4-29 Nucleotide sequence of 5'RACE fragment of <i>B. rotunda</i> <i>CHS</i> gene.....	120
Figure 4-30 Amino acid sequence of 5'RACE fragment of <i>B. rotunda</i> CHS protein .	121
Figure 4-31 Nucleotide sequence of 3'RACE fragment of <i>B. rotunda</i> <i>CHS</i> gene.....	122
Figure 4-32 Amino acid sequence of 3'RACE fragment of <i>B. rotunda</i> CHS protein .	123
Figure 4-33 Genomic PCR amplification of <i>B. rotunda</i> <i>CHS</i> gene using Initiation-Termination primers (RT-F, RT-R1).....	124
Figure 4-34 RT-PCR amplification of <i>B. rotunda</i> <i>CHS</i> cDNA using Initiation-Termination primers (RT-F, RT-R1).....	125
Figure 4-35 Complete sequence of <i>B. rotunda</i> <i>CHS</i> gene.....	127
Figure 4-37 Amino acid sequence of <i>B. rotunda</i> CHS protein.....	131
Figure 4-38 Complete sequence of <i>B. rotunda</i> <i>CHS</i> cDNA.....	133
Figure 4-39 Amino acid sequence of <i>B. rotunda</i> CHS protein.....	134
Figure 4-44 Hierarchical neural network analysis of <i>B. rotunda</i> CHS protein using SOMPA program.....	146
Figure 4-45 Predicted secondary structure of nine variants of <i>B. rotunda</i> CHS protein by Accelry Discovery Studio Client 2.5.....	147

Figure 4-46 Predicted secondary structure of variant 1 of <i>B. rotunda</i> CHS protein by Phyre version 0.2	148
Figure 4-47 Fold recognition of variant 1 of <i>B. rotunda</i> CHS protein using Phyre version 0.2.....	149
Figure 4-48 Alignment of variant 1 of <i>B. rotunda</i> CHS protein with c1xesA STS.....	151
Figure 4-49 Secondary structure of CHS from alfalfa (1CGZ) by Accelry Discovery Studio Client 2.5	152
Figure 4-50 Comparison of predicted secondary structure of <i>B. rotunda</i> CHS protein using Accelry Discovery Studio Client 2.5 and Phyre version 0.2 with secondary structure of alfalfa CHS	154
Figure 4-51 Ramachandran plot created by Accelrys Discovery Studio Client 2.5 for nine variants of <i>B. rotunda</i> CHS protein	156
Figure 4-52 Ramachandran plot created by PDBsum for nine variants of <i>B. rotunda</i> CHS protein	157
Figure 4-53 Three-dimensional structure of the predicted <i>B. rotunda</i> CHS protein in ball and stick form	158
Figure 4-54 Four CHS-specific conserved motifs in <i>B. rotunda</i> CHS protein.....	159
Figure 4-55 Significant conserved amino acids of <i>B. rotunda</i> CHS protein	162
Figure 4-56 Superimpose structure of significant amino acids of nine variants of <i>B. rotunda</i> CHS protein.....	167
Figure 4-57 Superimpose structure of all CHS clustering 95% to 1i88 with ligand binding	168
Figure 4-59 Superimpose structure of five variable amino acids of 5 [Å] away from the triad in variant 1 of <i>B. rotunda</i> CHS protein	170

Figure 4-60 Measurement of cavity volume of variant 1 of <i>B. rotunda</i> CHS protein and 1CGK through Pocket Finder	171
Figure 4-61 Docking of variant 1 of <i>B. rotunda</i> CHS protein with naringenin.....	173
Figure 4-62 Interaction of naringenin with variant 1 of <i>B. rotunda</i> CHS protein in the binding site.....	175

List of Abbreviation

3D	Three-dimensional
<i>B. pandurata</i>	<i>Boesenbergia pandurata</i>
<i>B. rotunda</i>	<i>Boesenbergia rotunda</i>
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	Naphtalene 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
<i>PKS</i>	<i>Polyketide Synthase</i>
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, cytotoxicity, 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.