MOLECULAR CLONING AND SEQUENCE ANALYSIS OF AN INULINASE GENE FROM AN ENDOPHYTIC FUNGUS

By

RABA'ATUN ADAWIYAH BINTI SHAMSUDDIN

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August 2010

Chairman : Shuhaimi bin Mustafa, PhD

Faculty : Biotechnology and Biomolecular Sciences

Endophytes, by definition, are organisms including fungi that live in close association with living plant tissues. Since no plants without endophytic fungi have been found to date, it is considered that endophytic fungi in plants are a common and general phenomenon. However, very few microorganisms living in plants were reported to be inulin degraders. Inulin occurs as a reserve carbohydrate in plants. Depolymerization of inulin involves the action of inulinase enzymes, isolated from microorganisms including fungi.

In this study, the endophytes producing inulinase were isolated from plant species in Serdang area. Thus, the fungal isolates were screened for inulin degrading enzymes. The results were evaluated in terms of substrate consumption and cell growth. All fungal isolates were able to degrade inulin and, among these, two isolates, named Asf1 and Onf1, were selected as the best inulinase producers. Genomic DNA of all isolates were extracted and amplified by polymerase chain reaction (PCR). A 1341 bp DNA fragment

containing inulinase gene was successfully amplified from Asf1 fungal isolate and was named as *inu2* gene in this study. Based on the morphological characteristics, Asf1 fungal isolate display closely-related to that genus of *Aspergillus*. The 18S ribosomal DNA sequence analysis revealed their high similarity of 99% to those of *Aspergillus* species. According to the neighbor-joining phylogenetic tree, Asf1 fungal isolate was shown to be closest to the species from the genus *Aspergillus.*

The complete sequence designated Asf1 *inu2* gene, was successfully obtained via rapid-amplification of cDNA ends-polymerase chain reaction (RACE-PCR). A 2.3 kb DNA fragment encoding endoinulinase, *inu2*, from Asf1 fungal isolate includes an open reading frame of 1,552 bp with calculated molecular weight of 55954.1 Da and signal peptide sequence of 23 amino acids. The deduced amino acid sequence of the Asf1 Inu2 showed 97%, 96%, 69% and 22% identities to that of *A. ficuum* inu2, *A. niger* inuB, *P. purpurogenum* and *K. marxianus*, respectively. A phylogenetic analysis showed that fungal endo- and exo-inulinases have indepently evolved the respective hydrolytic activities toward terminal and internal β-(2$\rightarrow$1)-fructofuranosidic linkages in inulin.

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

PENGKLONAN MOLEKUL DAN ANALISIS JUJUKAN GEN INULINASE DARIPADA KULAT ENDOFIT

Oleh

RABA'ATUN ADAWIYAH BINTI SHAMSUDDIN

Ogos 2010

Pengerusi : Shuhaimi bin Mustafa, PhD

Fakulti : Bioteknologi dan Sains Biomolekul

Endofit, sebagai definisi, adalah organisma termasuk kulat yang hidup melalui hubungkaitan rapat dengan tisu tumbuhan. Oleh kerana tiada tumbuhan tanpa endofit kulat ditemui sehingga kini, maka kewujudan endofit kulat di dalam tumbuhan dianggap sebagai satu fenomena biasa. Walau bagaimanapun, sangat sedikit mikroorganisma di dalam tumbuhan telah dilaporkan mendegradasikan inulin. Inulin adalah karbohidrat simpanan di dalam tumbuhan. Dipolimerisasi inulin melibatkan tindakan enzim inulinase, dipencilkan dari mikroorganisma termasuk kulat.

Di dalam kajian ini, endofit yang menghasilkan inulinase telah dipencilkan daripada spesis tumbuhan di kawasan Serdang. Endofit kulat yang dipencilkan diuji untuk penghasilan enzim inulinase. Keputusan ini dilihat dari segi penggunaan substrat dan pertumbuhan sel. Kesemua kulat yang dipencilkan mampu mendegradasikan inulin dan, di antaranya, dua pencilan yang dinamakan Asf1 dan Onf1, telah dikenalpasti sebagai penghasil inulinase terbaik. DNA genom bagi kesemua pencilan telah berjaya diekstrak

dan diamplifikasikan melalui tindakan rantai polimerase (PCR). Jujukan DNA sepanjang 1341 bp yang mengandungi gen inulinase telah berjaya diamplifikasikan daripada pencilan kulat Asf1 dan telah dinamakan sebagai gen *inu2* di dalam kajian ini. Berdasarkan kepada ciri morfologi, pencilan kulat Asf1 menunjukkan hubungan yang sangat rapat dengan genus daripada *Aspergillus*. Analisis jujukan DNA ribosom 18S memberikan 99% jujukan yang sepadan dengan *Aspergillus*. Berdasarkan kepada penyertaan jiran pokok filogenetik, pencilan kulat Asf1 menunjukkan hubungan yang sangat rapat dengan genus daripada *Aspergillus*.

Jujukan lengkap Asf1 gen *inu2* telah berjaya ditentukan melalui amplifikasi pantas hujung cDNA dan tindakan rantai polimerase (RACE-PCR). Jujukan DNA sepanjang 2.3 kb yang mengekodkan endoinulinase, *inu2*, daripada pencilan kulat Asf1 termasuk bingkai bacaan terbuka sepanjang 1,552 bp dengan kiraan berat molekul sebanyak 55954.1 Da dan jujukan isyarat peptida sebanyak 23 asid amino. Jujukan asid amino bagi Asf1 inu2 yang disimpulkan masing-masing menunjukkan identiti 97%, 96%, 69% dan 22% kepada *A. ficuum* inu2, *A. niger* inuB, *P. purpurogenum* dan *K. marxianus*. Analisis filogenetik menunjukkan endo- dan ekso-inulinase kulat mempunyai ketidakbergantungan terlibat dalam aktiviti-aktiviti hidrolitik masing-masing ke arah terminal dan dalaman rangkaian β-(2$\rightarrow$1)-fruktofuranosidik di dalam inulin.

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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:

Shuhaimi Mustafa, PhD
Associate Professor
Faculty of Biotechnology and Biomolecular Sciences
Universiti Putra Malaysia
(Chairman)

Yazid Abdul Manaf, PhD
Professor
Faculty of Food Science and Technology
Universiti Putra Malaysia
(Member)

Abdul Manaf Ali, PhD
Professor
Faculty of Agriculture and Biotechnology
Universiti Darul Iman Malaysia
(Member)

HASANAH MOHD GHAZALI, PhD
Professor and Dean
School of Graduate Studies
Universiti Putra Malaysia

Date:

DECLARATION

I declare the thesis is my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously, and is not concurrently, submitted for any other degree at Universiti Putra Malaysia or at any other institution.

RABA'ATUN ADAWIYAH SHAMSUDDIN

Date: 24 August 2010

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LIST OF ABBREVIATIONS

BLAST	Basic Local Alignment Search Tool
bp	basepair
cDNA	Complementary deoxyribonucleic acid
DEPC	Diethyl pyrocarbonate
DNA	Deoxyribonucleic acid
DNS	3,5 - Dinitrosalicylic acid
dNTPs	Deoxynucleotides
DTT	Dithiotreitol
E. coli	*Escherichia coli*
EDTA	Ethylenediaminetetraacetic acid
EtBr	Ethidium Bromide
GSP	Gene Specific Primer
HCL	Hydrochloric acid
IPTG	Isopropyl-β-D-thiogalactopyranoside
kbp	kilobasepair
kDa	kilo Dalton
KOH	Potassium Hydroxide
LB	Luria-Bertani
LD-PCR	Long Distance-Polymerase Chain Reaction
LiCl	lithium chloride
LN_2	liquid nitrogen

$MgSO_4$	magnesium sulfate
MMLV	Murime Moloney Leukemia Virus
MOPS	(3-[N-morpholino] propanesulfonic acid)
mRNA	messenger RNA
NaCl	sodium chloride
NaOH	sodium hydroxide
OD	Optical density
ORF	Open Reading Frame
PCR	Polymerase Chain Reaction
Poly A^+ RNA	Polyadenylated RNA
RACE	Rapid Amplification of cDNA End
RNA	Ribonucleic acid
RNase	Ribonuclease
rDNA	Ribosomal Deoxyribonucleic acid
rpm	revolution per minute
RT	Reverse Transcriptase
RT-PCR	Reverse transcriptase-Polymerase Chain Reaction
SDS	Sodium Dodecyl Sulfate/sodium lauryl sulfate
SMART	Switching Mechanisms At 5' End of RNA Transcript
TAG	Triacyglycerol
TE	Tris-EDTA
Tris	Tris[hydroxymethyl]aminomethana
Tris-HCL	Tris hydrochloride

U	Unit
UPM	Universal Primer Mix
UV	Ultraviolet
v/v	Volume per volume
w/v	Weight per volume
X-gal	5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside

CHAPTER 1

INTRODUCTION

Endophytes, by definition, are organisms that live in close association with living plant tissues. According to Cabral *et al*. (1993), endophytes are any fungi isolated from internal symptomless plant tissues. Since no plants without endophytic fungi have been found to date, it is considered that endophytic fungi in plants are a common and general phenomenon. Nowadays, endophytic fungi are of biotechnological interest due to their potential application as genetic vectors, as a source of secondary metabolites and as biological control agents. Fungal endophytes play important roles in ecosystem processes such as decomposition and nutrient cycling, and have beneficial symbiotic relationships with roots of many plants (Hyde and Soytong, 2008). However, only a few studies on endophytic fungi from Malaysian plant species have been conducted so far (Hazalin *et al*., 2009). Some endophytic fungi have been report to be inulin degraders to produce fructose or other oligosaccharides (Ohta *et al*., 2004).

Inulin is a widespread polyfructan with linear chains of β-(2→1)-linked fructose residues attached to a terminal sucrose molecule. Inulin occurs as a reserve carbohydrate in plants such as chicory, Jerusalem artichoke, asparagus and dahlia. Depolymerization of inulin involves the action of enzymes namely, inulinase. The inulinases are classified among the hydrolases and target on the β-(2→1)-linkage of inulin and hydrolyze it into fructose and glucose. They can be divided into exo-inulinases and endo-inulinase (Chi *et*

al., 2009). Exo- and endoinulinases, invertase (EC 3.2.1.26) and levanases (EC 3.2.1.65) are members of the superfamily *β*-fructofuranosidase and belong to family 32 in the numerical classification of glycosidase hydrolases (Pons *et al*., 1998).

Inulinase have been isolated from microorganisms including fungi. The genes encoding endo-inulinase were cloned from fungi such as *Penicillium purpurogenum* (Onodera *et al*., 1996), *Aspergillus niger* (Ohta *et al*., 2002) and *A. ficuum* (Uhm *et al*., 1998). Wen *et al*. (2003) reported cloning and sequencing of the inulinase gene of *Kluyveromyces marxianus* ATCC12424 and found an inulinase gene (*INU1*), which encoded a 555 amino acid precursor protein with a typical N-terminal signal peptide. Inulinase from microorganisms, especially filamentous fungi and yeasts, has potential applications in reducing production costs and improving syrup quality. Successful biotechnological application of inulinase clearly explains the increased attention being drawn to these enzymes (Ohta *et al*., 2002).

In this study, the microorganisms producing inulinase were isolated from plant species in Serdang area. Isolates were screened to determine the best inulinase producer and were identified by morphological characteristic, ribosomal DNA and phylogenetic analysis. For easy purification and expression studies, the cDNAs encoding inulinase gene from endophytic fungi was isolated and characterize. This results as the prerequisite before their potential can be exploited at the industrial level. The comparison of the available sequence data of the inulinases reported so far and presented in this work also was studied.

The objectives of this study are:

1. To isolate an inulinase gene from an endophytic fungus.
2. To screen the best inulinase producer.
3. To identify the endophytic fungi.
4. To clone and sequence the fungi inulinase gene.
5. To compare the available sequence data of the inulinases reported so far and presented in this study.

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