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Expression and Characterization of Thai Plant Glycosyl Hydrolases

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บทคัดย่อ (Thai abstract)

การผลิตรีคอมบิแนนท์โปรตีนเป็นขั้นตอนหนึ่งที่สำคัญในการศึกษาหน้าที่ของยีน แต่เป็นการยากที่จะผลิตโปรตีนของพืชหรือยูคาริโอตให้อยู่ในรูปที่ทำงานได้โดยใช้ระบบรีคอมบิแนนท์หรือแม้แต่การแยกโปรตีนออกมาจากสิ่งมีชีวิตกลุ่มนี้โดยตรง ในโครงการนี้เราได้พยายามสร้างระบบการผลิตรีคอมบิแนนท์โปรตีนของ β-glucosidases จากพะยูงและฉนวน (*Dalbergia* sp.) และการแยกโปรตีนออกมาให้บริสุทธิ์ ศึกษาการทำงานของเอนไซม์ พัฒนาการตัดต่อยีนและการผลิตรีคอมบิแนนท์โปรตีนของเอนไซม์พืช ในระบบต่างๆ ให้สะดวกและรวดเร็ว จากการทดลองในช่วงแรกที่ได้พยายามผลิต β-glucosidases ของ *Dalbergia* ใน *Pichia pastoris* โดยให้มี polyhistidine tags ต่ออยู่ที่ปลายคาร์บอกซี่และปลายอะมิโนของโปรตีน และให้มีการส่งโปรตีนที่ผลิตได้ออกนอกเซลล์ยีสต์ พบว่าตรงส่วนปลายของโปรตีนที่มี polyhistidine tags ต่ออยู่ถูกย่อยออกไป จึงได้แก้ปัญหานี้ด้วยการตัดกรดอะมิโนตรงปลายคาร์บอกซี่และปลายอะมิโนของโปรตีนออกไป และพบว่าการตัดกรดอะมิโนจำนวน 12 ตัวทางด้านปลายอะมิโนของเอนไซม์ dalcochinase ของ *D. cochinchinensis* ออกสามารถแก้ปัญหานี้ได้ จึงทำให้แยกโปรตีนออกมาให้บริสุทธิ์ด้วยวิธี immobilized metal affinity chromatography (IMAC) ได้ โปรตีนที่ผลิตและแยกออกมาให้บริสุทธิ์ได้ด้วยวิธีนี้มีสมบัติต่างๆ คล้ายกับ dalcochinase ที่แยกได้จากเมล็ด ก่อนหน้านี้เราได้ผลิตโปรตีนจาก cDNA ของ *D. nigrescens* ใน *E. coli* และ *P. pastoris* แต่โปรตีนที่ได้อยู่ในสภาพที่ไม่ทำงาน ดังนั้นจึงได้นำ cDNA ของ *D. nigrescens* ไอโซไซม์ที่ 2 ซึ่งให้ชื่อว่า dnbglu2 มาผลิตโปรตีนและพบว่าโปรตีนที่ได้อยู่ในรูปที่ทำงานได้ แต่ไม่สามารถแยกโปรตีนออกมาให้บริสุทธิ์ด้วยวิธี IMAC การตัดกรดอะมิโนทางด้านปลายอะมิโนของโปรตีนนี้ออกด้วยวิธีการเดียวกับที่ใช้กับ dalcochinase ทำให้แยกโปรตีน Dnbglu2 ออกมาให้บริสุทธิ์ด้วยวิธี IMAC ได้ โปรตีนนี้มีการทำงานแตกต่างจากเอนไซม์ที่แยกได้จากเมล็ด *D. nigrescens* เพียงเล็กน้อย เอนไซม์ของ *D. nigrescens* ที่ผลิตได้สามารถย่อย isoflavone 7-O-β-glycosides ได้ดีกว่า dalcochinase ของ *D. cochinchinensis* แต่ย่อย dalcochinin β-glucoside ซึ่งเป็นสับสเตรทของ dalcochinase ได้ต่ำกว่า dalcochinase เพื่อที่จะเพิ่มประสิทธิภาพการผลิตเอนไซม์ไกลโคซิเดสของพืชให้ดีและเร็วยิ่งขึ้นเราจึงได้พัฒนา Gateway cloning and expression system โดยการเปลี่ยนดีเอ็นเอพาหะ (expression vector) ที่ใช้ได้ผลดีในระบบการผลิตโปรตีนใน *E. coli* และ *P. pastoris* ได้แก่ pET32 และ pPICZαBNH8 ตามลำดับ ไปเป็น Gateway destination vectors และยังได้นำวิธีนี้ไปใช้กับดีเอ็นเอพาหะชนิดอื่นๆ ด้วย ได้ทดสอบระบบนี้กับ β-glucosidase ไอโซไซม์ Os4bglu12 ของข้าว จากการตัดต่อ cDNA ของ Os4bglu12 เข้ากับ pET32/DEST vector เพื่อผลิตโปรตีนใน *E. coli* สายพันธุ์ OrigamiB (DE3) และ pPICZαBNH8/DEST vector เพื่อผลิตโปรตีนใน *P. pastoris* พบว่าสามารถผลิตโปรตีนที่อยู่ในรูปที่ทำงานได้ปริมาณมาก ระบบนี้ได้นำมาใช้กับเอนไซม์ในกลุ่มไกลโคซิลไฮโดรเลส กลุ่มที่ 1 และ 35 ของข้าวไอโซไซม์ต่างๆ และสามารถผลิตโปรตีนที่อยู่ในสภาพที่ทำงานได้ได้สำเร็จเป็นจำนวนมาก และยังได้นำระบบนี้มาใช้ศึกษาผลของการกลายพันธุ์ของยีนของβ-galactosidase (*GalA*) ของมนุษย์ ด้วยการผลิตโปรตีนβ-galactosidase จาก cDNA ปกติและกลายพันธุ์ที่ถูกตัดต่อเข้าไปใน pPICZαBNH8/DEST vector ใน *P. pastoris* โดยสรุปโครงการนี้ทำให้เกิดการพัฒนาระบบการผลิตเอนไซม์ไกลโคซิเดสของยูคาริโอตในรูปแบบริคอมบิแนนท์โปรตีนเป็นอย่างมาก

Abstract:

Recombinant production of proteins is one of the most important steps in verification of gene functions, but it is often difficult to express active proteins from plants and other eukaryotes in recombinant systems and purify the proteins from them for characterization. In this project, we endeavored to create a system for recombinant expression and facile purification of isoflavone β-glucosidases from *Dalbergia* species, express, purify and characterize those enzymes, and develop a rapid system for cloning and expressing plant enzymes in various expression systems. Initial attempts at expression of *Dalbergia* β-glucosidases with C- and N-terminal polyhistidine tags as secreted proteins in *Pichia pastoris* suggested that proteolysis removed the tag on either end from the protein. To solve this problem, N- and C-terminal truncations were made and a 12-amino acid truncation from the N-terminus of *D. cochinchinensis* dalcochinase was found to allow expression of active protein that could be purified by immobilized metal affinity chromatography (IMAC). The protein expressed and purified in this way had similar properties to dalcochinase purified from seeds. Attempts to express the *D. nigrescens* cDNA that had previously been cloned in *E. coli* and *P. pastoris* resulted in only inactive protein. Therefore, a second cDNA, dnbglu2, was cloned and was found to produce active β-glucosidase/β-fucosidase in this system, which, again could not be purified by IMAC with C- and N-terminal polyhistidine tags. When the protein was truncated at the N-terminus in the same way as dalcochinase, Dnbglu2 could be expressed and purified by IMAC. This protein also had similar activity to protein purified from *D. nigrescens* seeds, though slightly different, and had higher activity toward isoflavone 7-O-β-glycosides than *D. cochinchinensis* dalcochinase, but lower activity on the dalcochinase substrate, dalcochinin β-glucoside. In order to make expression of plant glycosidases in our lab more efficient, we also developed a Gateway cloning and expression system by converting our most successful *E. coli* and *P. pastoris* expression vectors, pET32 and pPICZαBNH8, respectively, to Gateway destination vectors and acquiring several other expression vectors. This system was tested on the rice β-glucosidase gene Os4bglu12 and found to produce high amounts of active protein from the pET32/DEST in the OrigamiB (DE3) strain of *E. coli* and from the pPICZαBNH8/DEST construct in *P. pastoris*. Since then, several other glycosyl hydrolase family 1 and 35 genes from rice have been successfully expressed. The system also provided a rapid way to assess the effect of a mutation in the *GalA* gene by expressing the human β-galactosidase from the mutant and normal cDNA in *P. pastoris* media with the pPICZαBNH8/DEST plasmid. Thus, this project allowed considerable advancement in eukaryotic glycosidase expression in recombinant systems.

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List of Abbreviations and Symbols

A	Absorbance
Amp	Ampicillin
ATP	Adenosine triphosphate
bp	Base pairs
BSA	Bovine Serum Albumin
°C	Degree celsius
cDNA	Complementary deoxynucleic acid
CTP	Cytosine triphosphate
DEPC	Diethyl pyrocarbonate
DNA	Deoxyribonucleic acid
dNTPs	dATP, dCTP, dGTP and dTTP
EDTA	Ethylene diamine tetraacetic ccid
g	Gravitational acceleration
GH	Glycosyl hydrolase(s)
GH1	Glycosyl hydrolase family 1
GH5	Glycosyl hydrolase family 5
GH35	Glycosyl hydrolase family 35
GlcNAc	N-acetyl glucosamine
GTP	Guanidine triphosphate
(m, n) g	(Milli, Nano) gram
h	Hour
HPLC	High performance liquid chromaography
IPTG	Isopropyl-β-D-thiogalactopyranoside
kDa	Kilo Dalton
(m, μ) L	(Milli, micro) Liter
min	Minute
(m, μ, n) M	(Milli, micro, nano) Molar
(μ, n, pmol) mol	(Micro, nano, pico) Mole
mRNA	Messenger ribonucleic acid
Mw	Molecular weight
4MUGlc	4-Methylumbellriferyl-β-D-glucoside
OD	Optical density

List of Abbrevations (continued)

PAGE	Poly acrylamide gel electrophoresis
PCR	Polymerase chain reaction
pI	Isoelectric point
*p*NP	*para*-Nitrophenol
*p*NPFuc	*para*-Nitrophenol-β-D-fucoside
*p*NPGlc	*para*-Nitrophenol-β-D-glucoside
RNA	Ribonucleic acid
RNase	Ribonuclease
SDS	Sodium dodecyl sulfate
s	Second
SSC	Saline sodium citrate
TEMED	Tetramethylenediamine
Tris	Tris-(hydroxymethyl)-aminoethane
TTP	Thymidine triphosphate
UV	Ultraviolet
U	Unit, µmol/min
v/v	Volume/volume
w/v	Weight/volume

Section I: Introduction

1.1. Importance and Background of Reseach Problem (ความสำคัญและที่มาของปัญหาการวิจัย)

Background and literature survey

Many chemical species found in plants, have been found to be glycosides. That is, they include one or more sugar residues as a part of their structure. Common glycosides of economic interest include vitamins, phytohormones, pigments, lignin precursors, and a broad range of other compounds (Hösel and Conn, 1982; Esen, 1993). These natural glycosides often have properties of medicinal interest in addition to their roles in the plant (Yoshikawa et al. 1998). Plants that contain such glycosides often contain enzymes which remove the sugars: glycosidases (King and Davies. 1995; Rubinelli, Hu, and Ma, 1998; Smith, Starrett, and Gross, 1998). Glycosidases that have been found in Thai plants include: α-D-mannosidases, α-D-N-acetylglucosidases, α-D-galactosidases, β-D-fucosidases, β-D-glucosidases (Surarit et. al., 1995) and chitinases (Kaomek et al., 2003).

Many of these enzymes function in defense against herbivores and parasites in other plants (Falk and Rask L. 1995). Many plant β-glucosidases, for instance, hydrolyze glycosides to produce toxic substances upon disruption of their tissue integrity, such as linamarinase in cassava, which produces a cyanogenic product (Dunn, Hughes, and Sharif, 1994). Similar β-glucosidases play the same role in other plants (Cicek and Esen, 1998). Chitinases have been found to confer fungal resistance, suggesting they slow fungus by hydrolyzing the chitin in their cell walls (Collinge et al. 1993, Grison et al., 1996). Chitinases have also been shown to have a potential role in modulating legume-*Rhizobium* interactions, while isoflavone β-glucosidases are thought to be important to release of isoflavones to attract *Rhizobium*. Soybean chitinase can inactivate NOD factor, a glycoside which is produced by Rhizobium to cause the legumes to make nodules in which the *Rhizobium* live (Collinge et al. 1993).

Aside from defense, glycosidase, such as β-glucosidases, play important roles in lignification, phytohormone activation, stress response, cell wall remodeling and scent volatilization (Opassiri et al., 2006). Monolignol releasing β-glucosidases have been isolated from pine and *Arabidopsis thaliana* (Dharmawardhana et al., 1995, Escamilla-Treviño et al., 2006). In *A. thaliana*, a drought-stress activated β-glucosidase hydrolyzes abscissic acid (AA) glucosyl ester to modulate active AA levels (Lee et al., 2006). Other β-glucosidases have been found to hydrolyze cytokinin glucosides (Falk and Rask, 1995) and gibberellin glucoside (Schlieman, 1985). β-Glucosidases hydrolyzing oligosaccharides released from cell wall β-glucans have been characterized in barley and rice (Hrmova et al., 1996; Akiyama

et al., 1998; Opassiri et al., 2003, 2004). So, these enzymes play many important roles in plants, suggesting that increased understanding of their activities may help with crop improvement.

Glycosidases also have many applications of current or potential economic significance to Thailand. For instance, since vitamins in plants are often glycosides, animal feeds derived from plants may be treated with β-glucosidases and other glycosidases to improve nutritional availability of the vitamins (Gregory, 1998). Additionally, resveratrol, a substance associated with health benefits found in red wines may be increased in the wine by addition of β-glycosidases. (Gonzalez-Candelas et. al., 2000). Chitinases are not only valued for their anti-fungal properties, but also for use in producing chito-oligosaccharides from shrimp carapace and other chitinous waste. Beta-galactosidases have also been used to treat milk to convert lactose to galactose and glucose, which may improve the taste and texture of some dairy products. Because of these applications, large-scale production and engineering of these enzymes for better function is of interest. Though microbial sources of the enzymes have been more exploited to date, the somewhat different properties of plant enzymes may be advantageous in some cases.

These glycosidases fall into several families of evolutionarily related proteins (Henrisatt, 1991; Henrisatt and Bairoch 1993, 1996; Henrisatt and Coutinho, 1999). For instance, plant β-glucosidases mainly belong to glycosyl hydrolase family 1, and beta-galactosidases to family 35. The evolutionary relationships among these enzymes allow us to predict the structures and amino acids that are critical to catalytic properties from known structures (Barrett et al, 1995, Sanz-Aparicio et al, 1998; Czjzek et al., 2000, 2001).

Using the evolutionary relationship between these enzymes, we have cloned cDNA for β-galactosidase, β-glucosidase, chitinase from various plants (Ketudat Cairns et al., 1999, 2000; Kaomek et al., 2003). In related work with the Biochemistry Laboratory of Chulabhorn Research Institute, we have previously cloned a novel rotenoid β-glucosidase from *Dalbergia cochinchinensis* Pierre, which had a potentially useful substrate (Svasti et al., 1998; Ketudat-Cairns et al., 2000). This enzyme has also been shown to be useful for synthesis of β-glucosides of various alcohols (Gonsales-Candelez, 2000). Before this project began, a closely related β-glucosidase from *Dalbergia nigrescens* had also been cloned (Ketudat Cairns et al., 2001). This enzyme was 87% identical at the amino acid level to the *D. cochinchinensis* β-glucosidase and the substrate-specificity is similar, but there are significant differences and the major natural substrates are not the same. The residues previously described to be important in sorghum dhurrinase and maize β-glucosidase isozymes substrate

specificity are mostly not different in these 2 enzymes (Cicek et al., 2000, Czjzek et al., 2000, 2001). However, this enzyme could not be expressed in active form, so a second cDNA was cloned in this project. This enzyme was less similar to *D. cochinchinesis* dalcochinase (around 81% identical), and could not hydrolyze dalcochinin glucoside efficiently. Therefore, these enzymes are a good model to explore the basis of substrate-specificity further. When this project began, we had previously expressed recombinant *D. cochinchinensis* enzyme in *P. pastoris*, however the low yields made it difficult to completely characterize and think of engineering the enzymes. During this project, we developed an improved *P. pastoris* expression system for this enzyme (Toonkool et al., 2006) and applied this system to express an active isoflavonoid β-glucosidase from *D. nigrescens*. We have also worked to express β-glucosidases and β-galactosidases from rice to characterize their functions and potential usage (Opassiri et al., 2003, 2006; Chantarangsee et al., 2007). Optimization of expression of all these enzymes will facilitate exploring their function and potential for application.

1.2. Research Objectives (วัตถุประสงค์ของการวิจัย)

1. 2.1.2.1. Establishment of efficient recombinant expression systems for β-glucosidases from *Dalbergia* sp and other glycosidases we have previously cloned.
2. Development of robust, efficient methods for purification of the recombinant enzymes.
3. Further characterization of the functional (enzymatic) properties of the enzymes and their applications.
4. To determine the amino acids that contribute to substrate-specificity in the enzymes by mutagenesis.

1.3. Scope of Research (ขอบเขตของการวิจัย)

Due to the limitation on funding, the project concentrated on expression of a few enzymes and on developing a system for rapid screening of protein expression in different vector/host systems. The enzymes for which expression was optimized were *Dalbergia cochinchinensis* dalcochinin-glucoside β-glucosidase (dalcochinase, Thai rosewood β-glucosidase) and *D. nigrescens* Dnbglu2 isoflavone β-glucosidase. The rapid screening system was set-up with Gateway technology with conversion of pET32a vector, aquiring other *E. coli* expression vectors, and conversion of pPICZα-derived vectors for Gateway technology. This system was used to express rice glycosyl hydrolase family 1 and 35 genes in a project funded from another grant (BIOTEC), and to express normal and mutant human α-galactosidase from *GalA* cDNA. Characterization, mutagenesis and characterization of mutants had to be done on other grants due to insufficient funding on this grant.

1.4. Short Description of Methods (ข้อตกลงเบื้องต้น)

This project involved the optimization of an expression system for *D. cochinchinesis* dalcochinase to allow simplified purification, cloning and expression of a *D. nigrescens* β-glucosidase, and development of a Gateway expression system and expression of some genes of interest in that system. In the process, several vectors and cDNA had to be modified to allow expression and purification.

For expression of dalcochinase, several modifications were attempted, including C-terminal mutagenesis and shortening to try to prevent proteolysis between the catalytic domain of the protein and the C-terminal portion of the gene, production of pPICZα vector with N-terminal His-tags with (pPICZαBNH) and without

(pPICZαBNH8) an enterokinase protease site, and truncation of the 5' of the cDNA fragment to remove protease sites from the N-terminus of the protein. Expression of the *D. nigrescens* β-glucosidase started with mutagenesis of the cDNA of *Dnbglu1* (the initial clone reported in a previous grant) to try to produce active protein, cloning of a new cDNA, *Dnbglu2*, which was able to express active protein; and cloning of this cDNA with the same N-terminal truncation as the dalcochinase into pPICZαBNH8, followed by expression and fermentation in *Pichia pastoris* to produce the protein. Subsequently, the dalcochinase could be mutated to be more similar to Dnbglu2 to test for the determinants of substrate specificity differences between these enzymes.

The production of a Gateway expression system for screening of appropriate expression systems for expression of the proten was done by aquiring vectors and converting favored vectors in the laboratory to generate Gateway destination expression vectors. The vectors obtained included the pBAD/DEST49 for ARA /BAD promoter regulated expression of thioredoxin fusion proteins from Invitrogen, the pMAL vectors for production of maltose-binding protein fusion proteins from the Salk Institute Structural Biology Group, and the Curtis expression vectors for expression in plants (Curtis and Grossniklaus, 2003) from the University of Zürich. We were able to convert the pET32a vector for T7-regulated expression in *E. coli*, and the pPICZαBNH and pPICZαBNH8 vectors for expression in *P. pastoris* to Gateway vectors, which allowed expression of many proteins in these systems, including several rice family 1 and family 35 glycosyl hydrolases and human α-galactosidase A.

1.5. Benefits and output from this research project (ประโยชน์ที่ได้รับจากการวิจัย)

From the work on optimizing protein expression of *Dalbergia* β-glucosidases, we were able to contribute to two publications (Toonkool et al., 2006; Chuankhayan et al., 2007b), in addition to the training of Ph.D. students and research assistants. Dr. Phimonphan Chuankhayan and Dr. Pornphimol Metheenukul were able to complete their Ph.D. projects, which included work on this project. In addition, Ms. Narumol Mothong was able to learn about protein expression methods. Thus, this project contributed to both the scientific knowledge of protein expression and legume isoflavone β-glucosidase function and to the scientific output and human resources development of Thailand.

Production of the Gateway system greatly facilitated the work on other grants in characterizing enzymes from rice, as well as allowing us to express normal and mutant GalA

cDNA to produce the respective human α-galactosidase in *P. pastoris* and show that the mutation indeed affected the activity of the protein. This contributed to three publications on human and rice enzymes (Wattanasirichaigoon et al., 2006; Opassiri et al., 2006, 2007). The enzymes produced in this system subsequently served to contribute to the theses of several M.Sc. and Ph.D. students and will continue to contribute to papers in the future. This again points to its importance in production of knowledge, human resources and capacity building.

2. Materials and Methods

2.1 Materials.

Dalbergia nigrescens Kurz seeds were also collected at Suranaree University of Technology. RNA was extracted seeds after soaking in water overnight and and germinating on sterile tissue paper or from immature seeds that were near their final size, but still green in color.

SuperScript reverse transcriptase II, RNase H, Gateway vectors, Gateway conversion kit, pENTR-D/TOPO, pENTR4, pPICZαB, pTOPO-BLUNT, RL Clonase, BP Clonase and Trizol reagent were from Invitrogen (Carlsbad, CA, USA). Oligonucleotides were ordered from Geneset/Proligo Oligos Ltd. (Singapore), though some *D. cochinchinensis* dalcochinase-specific primers were graciously provided by Prof. Dr. MR Jisnuson Svasti. The pMalc/DEST, pMals/DEST and pThio/DEST plasmids were kindly provided by the Structural Biology Group of the Salk Institute. *Para*-nitrophenyl glycoside substrates, and other commercial glycosides, aside from isoflavone glycosides and oligosaccharides, were products from Sigma, Fine Chemicals (St Louis, MO, USA). Restriction enzymes, deoxyribonucleotides, 5-Bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal), pGEM-T plasmid, *Taq* polymerase, *Pfu* DNA polymerase, T4 DNA ligase, and Poly Tract mRNA isolation system IV were products from Promega (Madison, WI, USA). pT7blue, pET-23d, pET-32a and pET40a plasmids, BL21 (DE3), Origami (DE3) and OrigamiB (DE3) *E. coli* were from Novagen (Madison, WI, USA). pUC19 plasmid, phage λgt11, and the 5′ RACE kit were from Takara (Toyoko, Japan). The IMAC resin for self-charging was from GE Amersham Pharmacia Biotech (Uppsala, Sweden).

D. nigrescens isoflavone glycosides, dalpatin β-D-apiosyl-6-O-β-D-glucoside and dalnigrecin β-D-apiosyl-6-O-β-D-glucoside, were purified according to the method of Chuankhayan et al., 2005, while dalochinin β- D-glucoside was expressed as described below. Dalcochinin β-D-glucoside was purified from *D. cochinchinensis* seeds by ethanol extraction. Ten grams of seed powder was stirred overnight with ethanol at room temperature. The ethanol was removed by drying, and the solid residue was extracted with hexane 2-3 times and redissolved with methanol. This crude extract was separated on an LH-20 column with methanol as eluent. The pooled dalcochinin glucoside fraction was further purified by HPLC on an XDB-C_{18} reverse phase column eluted with 38% methanol on an Agilent 1100 series

HPLC with detection by absorbance at 260 nm wavelength on a diode array detector. The dalcochinin glucoside peak was collected and dried by speed vacuum. The mass of the purified glycoside was verified to match dalcochinin β-D-glucoside [15] by electrospray mass spectrometry ($[M+Na]^+ = 597.13$).

2.2 Enzyme assays.

Assays for β-glucosidase, β-fucosidase and other glycosidase activities were tested by hydrolysis of *p*-nitrophenol (*p*NP) glycosides in 0.1 M sodium acetate, pH 5.0 for dalcochinase and initial rice glycosidase studies and pH 5.5 for *D. nigrescens* for 10 min, as described by Surarit et al (1995). To test hydrolysis of natural glucosides, enzyme was incubated with the substrate for 10 min, as above, and the glucose released was quantitated with a PGO glucose oxidase assay (Sigma Fine Chemicals). For soy isoflavone glycosides, the glucose was detected in this manner, but with a reduced PGO development time (15 min) to avoid interference by the isoflavone aglycones or the aglycones were detected by HPLC according to the method of Chuankhayan et al. (2007a).

For α-galactosidase activity, the media of *P. pastoris* that had been induced to produce recombinantly expressed protein was sent to the Biochemistry Laboratory of Chulabhorn Research Institute for analysis. The α-galactosidase enzyme was assayed with methylumbelliferyl α-D-galactoside as substrate and N-acetylgalactosamine as inhibitor of Gal B, as described (Kusiak et al., 1978), except that the reaction volume was reduced to 70 μL to allow fluorescence measurement with a microtiterplate reader (Wattanasirichaigoon et al., 2006). Protein was determined by the method of Bradford with bovine albumin as standard (Bradford, 1976).

2.3. Plasmids for tagged expression in *P. pastoris.*

Three modified pPICZαB *P. pastoris* expression vectors were used for this study, one with a thrombin site before the C-terminal tag (pPICZαB-Thrombin) and two with N-terminal tags of 6 histidine residues and an enterokinase site (pPICZαBNH) and 8 histidine residues (pPICZαBNH8). The pPICZαB-Thrombin was provided by Mariena Ketudat-Cairns group, but was made by a similar strategy, so it is also explained. The modified pPICZαB plasmids were made by the following strategy. First, two complementary oligonucleotides, Thrombin_sense and Thrombin_antisense (Table 1) for pPICZαB-Thrombin, PICZαBNHF and PICZαBNHR for pPICZαBNH, and PICZαBNH8F and PICZαBNH8R (Table 1) for pPICZαBNH8, were annealed by heating to 95-100°C and slowly cooling to room

temperature. The annealed thrombin oligonucleotides were ligated into the *Sac*II-*Xba*I sites of the pPICZαB vector, creating pPICZαB-Thrombin by standard methods (Maniatis et al., 1982). The pPICZαBNH, corresponding to a sequence encoding 6 histidine residues, an enterokinase site, and *Pst*I, *Sna*BI and *Eco*RI sites, and pPICZαBNH8 hybrid, corresponding to the coding sequence of 8 histidine residues, followed by the *Pst*I, *Sna*BI and *Eco*RI sites, were cloned between the *Pst*I and *Eco*RI sites of pPICZαB.

Table 1 Primers used for construction of *P. pastoris* vectors.

Primer name	Primer sequence
PICZαBNHF	5′-CAT CAC CAT CAT CAC CAT GAC GAC GAC GAC AAG GCT GCA GTA-3′
PICZαBNHR	5′-AATTCTACGTACTGCAGCCTTGTCGTCGTCGTCATGATGGTGATGGTGATGTGCA-3′
PICZαBNH8F	5′-CAT CAC CAT CAC CAT CAT CAC CAT GCT GCA GTA CGT AG-3′
PICZαBNH8R	5′-AAT TCT ACG TAC TGC AGC ATG GTG ATG ATG GTG ATG GTG ATG TGC A-3′
Thrombin_sense	5′-GGT TGG TTC CTA GGG GTT CTA TT-3′
Thrombin_antisense	5′-GGC GCC AAC CAA GGA TCC CCA AGA TAA GAT-3′

2.4. Thai rosewood β-glucosidase cDNA construction in pPICZαB plasmid and expression

The pPICZαB-thrombin vector was selected to express and secrete recombinant proteins in *P. pastoris*. Proteins were expressed as fusions to an N-terminal peptide encoding the *S. cerevisiae* α-factor secretion signal and some also included a C-terminal peptide containing a thrombin cleavage site, the *myc* epitope for detection and a polyhistidine tag for purification on metal chelating resin.

pPICZαB-Thrombin-PYG(R519W) was constructed to incorporate a cDNA encoding a mature Thai rosewood β-glucosidase with a mutation of Arg519 to Trp into pPICZαB-Thrombin. The cDNA was amplified from the previously described pPIC9K expression construct (Ketudat-Cairns et al., 2000) with the DalYEXf1 and PMCterm1 primers (Table 2) and cloned into the pPICZαB-thrombin vector between the Pst1 and Xba1 sites. The resulting pPICZαB-Thrombin-PYG (R519W) was transformed into *P.pastoris* GS115, and colonies were screened for enzyme production by small scale expression. Small scale cultures from 14 colonies were induced in 2 mL BMMY with 0.5% methanol and cultured 30°C for 3 days with 0.5% methanol added for induction each 24 h. The level of recombinant protein expression was followed day by day. The transformed clone which gave highest activity was selected for more expression. The culture medium was collected and subjected to IMAC, to determine if it would bind for purification.

Table 2 Primers used for amplification and sequencing of the Thai rosewood β-glucosidase cDNA

Primer name	Primer sequence
DalYEXf1	5′-CTTTAAGCTTATGCTTGCAATGACATC-3′
DncVPPFpst1f	5′-CAT TCC TGC AGT TCC TCC ATT CAA CCG AAG-3′
DcR488StopXbar	5′-CCCCTAGACTTAACGTGCCAGAAAATACTTGAAC-3′
PMCTERM1	5′-AAG ATC TAG ATC AAA AGC CTT CAA TGC CTC TC-3′
5′-AOX	5′-GACTGGTTCCAATTGACAAGC-3′
3′-AOX	5′-GCAAATGGCATTCTGACATCC-3′

2.5. Construction of C-terminally truncated Thai rosewood β-glucosidase cDNA in pPICZαB plasmid and expression

The Thai rosewood β-glucosidase was truncated at arginine 488 to reduce C-terminal protease susceptible sequence. The insert for pPICZαB-Truncated R488 was amplified from the DalYEXf1 and DcR488StopXbar primers (Table 2) with *Pfu* polymerase and pPICZαB-Thrombin-PYG (R519W) as template. pPICZαB-Truncated R488 was constructed from a cDNA amplified with DalYEXf1 and DcR511StopXbar primers (Table 2) with *Pfu* polymerase and pPICZαB-Thrombin-PYG(R524W) as template. The PCR condition was 95°C, 1 min, 55°C, 1 min, and 72°C, 2 min for 30 cycles. The PCR product was ligated into *Sma*I digested pBluescript SK(+) and transformed into *E.coli* DH5α. The transformed colonies were selected by blue-white colony screening. The recombinant plasmids were extracted by alkaline lysis and checked by digestion with *Pst*I and *Xba*I. The insert digested and ligated with pPICZαB to produce the construct named pPICZαB-Truncated R488. The corrected plasmid was digested with *Pst*I and *Xba*I and ligated with *Pst*I and *Xba*I digested pPICZαB, named pPICZαB-truncated R488. The pPICZαB-truncated R488 was linearized by *Sac*I and then transformed into *P. pastoris* GS115.

2.6. Construction of N-terminally tagged Dalcochinase expression vector.

Constructs for expression of dalcochinase with an N-terminal polyhistidine-tag were then made. The dalcochinase cDNA was PCR amplified with the DalYEXf1 and PMCTERM1 primers, cloned into pGEM-T easy vector, excised, and inserted between the *Pst*I and *Sac*II sites of the p PICZαBNH and pPICZαBNH8 vectors to create pPICZ-His_6-TRBG and pPICZ-His_8-TRBG. A second N-terminally His-tagged protein expression construct was made with a truncated N-terminus. The dalcochinase cDNA clone was

amplified with the trncTRBGF and PMCTERM1 primers, and cloned into pPICZαBNH8, as described for pPICZ-His_8-TRBG, to create pPICZ-His_8-trncTRBG.

2.7. Transformation and expression of the *P. pastoris* strain (*his4*)/GS115

pPICZαB-Thrombin-PYG(R519W), pPICZαB-Truncated R488 and pPICZαB vector (negative control) were digested with *Sac*I before transformation by the electroporation method (Invitrogen). Transformed colonies were used to inoculate 10 ml of buffered minimal glycerol-complex medium (BMGY), pH 6. After 1 day at 200 rpm and 30 °C, the cells were pelleted and resuspended in 2 ml buffered minimal methanol-complex medium (BMMY). Following another 3 days at 30 °C,

The expression was started by inoculation of each transformed colony into 10 mL of BMGY and incubating the culture at 30°C, 250 rpm overnight, until the culture reached an OD_{600} of 2-3. The cultured yeast was collected by centrifugation and transferred to the volume of BMMY with methanol that would give a total culture OD_{600} = 20. The enzyme activity and OD_{600} were determined from collected media. At appropriate time points, the culture was centrifuged at 4000 rpm for 5 min at room temperature and the amount of recombinant proteins in the supernatant was estimated by activity assays using *p*NP-β-D-glucopyranoside (*p*NPGlc) and *p*NP-β-D-fucopyranoside (*p*NPFuc) as substrates. The transformed clones which gave the highest activity were selected for recombinant Thai rosewood β-glucosidase expression.

2.8. Construction of expression vectors for D. nigrescens Dnbglu2 in *P. pastoris*.

Cloning of the Dnbglu2 cDNA

The total RNA was isolated from immature seeds collected from a *D. nigrescens* tree 2-3 months after flowering. Then, 0.1 g of immature seeds was ground in liquid nitrogen and extracted with Trizol reagent (GIBCO-BRL, Invitrogen) according to the manufacturer's instructions. The total RNA pellet was resuspended in DEPC-treated water, and an aliquot (1-5 μg) was used as template for first strand cDNA synthesis catalyzed by Superscript II Reverse Transcriptase (Invitrogen) with Q_T primer (Frohman, 1993), as recommended by Invitrogen.

The first-strand cDNA was used as template to amplify cDNA fragments with several combinations of the For.2, For.3, For.4, For.5, For.6, Rev.4, Rev.5, Rev.7 and Rev.8 primers designed from the *D. cochinchinensis* β-glucosidase cDNA sequence by Ketudat Cairns et

al., (2000). The amplifications were performed using *Taq* polymerase, and the PCR products were gel purified and cloned into pGEM-T Easy vector (Promega) according to the supplier's recommendation. The nucleotide sequences of single clones were determined by automated sequencing with the ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems, Foster City, CA). The sequence of the initial cDNA clones was used to design new specific primers, PBGF1 (5′-CACCATTTGTAACCATTTTTCATTG-3′) and PBGrev2 (5′-GTAAAGCAACAAATCTCGAAGTCC-3′), which were used in PCR to amplify a new fragment from germinating seed cDNA. This fragment was cloned and sequenced as described above, and designated the *Dnbglu2* cDNA.

The rapid amplification of cDNA ends (RACE) technique was used to amplify cDNAs from the 3′and 5′ends of the mRNA with the Q_T, Q_o, and Q_i end primers described by Frohman, (1993). For 3′ RACE, the first strand cDNA was synthesized from the Q_T primer and total *D. nigrescens* seed RNA as described above and used as template for nested PCR. For the *Dnbglu1* cDNA, the first PCR reaction was done with *Taq* polymerase and the Q_o and PTDnF1 (5′-GGTGGCTTCTTAGATCGTAG-3′). Then, the PCR product was used as a template with the Q_i and PTDnF1 primers in a second PCR amplification. The 5′ RACE was done by anchoring a poly A tail sequence at the 3′ end of the first strand cDNA that had been reverse transcribed from the PTDnR1 (5′-GCCATTTGTGGTGAAGACTTG-3′) primer with dATP and Terminal Deoxynucleotidyl Transferase (TdT) according to the supplier's instructions (Promega). The anchored cDNA was used as template in a PCR reaction with *Taq* polymerase, the Q_T and PTDnR1 primers. A second amplification was performed with the product and the PTDnR2 (5′-CGAAAATCATTTACAACCCTAC-3′) and Q_o primers. The RACE PCR products were cloned and sequenced, as described for the initial PCR products. The sequences of the 3′ and 5′ RACE products were used to design the 3′ and 5′ terminus primers. The full length CDS *Dnbglu2* cDNA was amplified with the Dn2_3′UTRr1 (5′-AAATGTACCAAAGCCACAAAC-3′) and Dn2_5′UTRf1 (5′-TCCTT TCTTTCATCTCATGATTG-3′) primers and cloned and sequenced as described above.

Expression vector construction

Initial clones were amplified with the DN2NTERMPstIf (5′-ATTCCTGCAGTTCCTCC ATTCAATCGAAG-3′) and DN2CTERMXbaIr (5′-ATCAAATGCTTGAATGGCCCACTT-3′) primers with the full-length cDNA as template and *Pfu* polymerase to generate the coding sequence for the predicted mature Dnbglu1 protein with the PstI and XbaI sites for cloning

into pPICZαB-Thrombin, pPICZαBNH and pPICZαBNH8. The PCR product was cloned into pZeroBlunt/TOPO according to the manufacturer's instructions (Invitrogen). The resulting plasmid was cut with PstI and XbaI and the insert gel purified and cloned into pPICZαB-Thrombin. The insert was sequenced and was subsequently excised from this plasmid and cloned into pPICZαBNH and pPICZαBNH8.

The DnVPPFPstI (5′-GTGCAACCATTCCTGCAGTTCCTCCATTCAATCGAAG-3′) and Dn2CTERMXbaIr (5′-ATCAAAATGCTTGAATGGCCCACTT-3′) primers were used in PCR to introduce the desired *Pst*I site at the start of the sequence encoding the protein starting from the same position successful for dalcochinase expression with an N-terminal His-tag (below and Toonkool et al, 2006) and an *Xba*I site at the 3′ end of the coding region. The reaction was done with a 1:1 unit mixture of Hot Star *Taq* and *Pfu* polymerases. Then, the PCR product was gel purified, reamplified with *Pfu* polymerase and cloned into Zero Blunt® TOPO PCR cloning vector (Invitrogen). The clones containing insert were digested with *Pst*I and *Xba*I, and the insert was gel purified and cloned into the pPICZαNH_8 plasmid.

2.9. Sequence analysis

Initial analysis of DNA sequences were done with BLAST (Altschul et al., 1997) at the National Center for Biotechnology Information (NCBI: http://www.ncbi.nlm.nih.gov). The N-terminal signal sequence was predicted with SignalP (Bendtsen et al., 2004) and protein properties were predicted with the programs available at Expasy (http://www.expasy.org). *Dalbergia* β-glucosidases and related protein sequences were aligned with ClustalX, an implementation of ClustalW (Jeanmougin et al., 1998; Thompson et al., 1994). The alignments were adjusted with Genedoc, and the N-terminal signal sequence and nonconserved region were removed prior to phylogenetic analysis by the neighbor-joining tree implementation of ClustalX.

2.10. Recombinant expression and purification of Dnbglu2

The selected plasmid clones were linearized with *Sac*I restriction enzyme and then transformed into *P. pastoris* strain GS115 or YM11430 by electroporation. After transformation into *P. pastoris* strain GS115 for small-scale expression, the selected clones were expressed in BMGY medium and induced in BMMY medium with 1% methanol induction as described in the *Pichia* manual (Invitrogen). The media were tested for *p*-nitrophenyl-β-D-fucopyranoside (*p*NP-Fuc) activity using 5 μL of media incubated with 1 mM *p*NPF in 0.1 M sodium acetate, pH 5.0, 30 °C for 10 min. Then, the reaction was stopped

with 2 volumes of 2 M Na_2CO_3 and the absorbance of the released *p*-nitrophenol (*p*NP) was measured at 405 nm. Once a construct was determined to produce active enzyme, it was expressed in YM11430 in the same way, and clones with highest levels of enzyme activity in the media were selected. These clones were expressed in a 1-L culture in a 2-L fermentor by methanol-limited fed-batch fermentation in defined media, as previously described by Charoenrat et al. (2005). The media was dialyzed and concentrated. Recombinantly expressed enzymes were purified from desalted, concentrated fermentor media with Talon Co^{2+} immobilized metal affinity chromatography (IMAC) resin, according to the manufacturer's protocol (Clontech, Mountain View, CA, USA).

2.11. *β*-Glucosidase protein analysis

Protein concentration was determined by the Lowry method (Lowry *et al.*, 1951) with bovine serum albumin as standard, while column effluents were screened for protein by measurement of A_{280}. Denaturing or non-denaturing gel electrophoresis was run according to the general method of Laemmli (1970). SDS-denaturing polyacrylamide gel electrophoresis was performed on 10% polyacrylamide separating gels with Bio-RAD Low-range protein markers (Bio-RAD, Corp., Hercules, CA, USA), and stained with Coomassie Brilliant Blue R250. Non-denaturing polyacrylamide gel electrophoresis (activity gels), was performed with 5% polyacrylamide stacking gels and 7% polyacrylamide separating gels in *Laemmli* buffer without SDS. The gel was stained separately for β-glucosidase, β-fucosidase and β-galactosidase activity using 1 mM 4-methylumbelliferyl-β-glycosides. The fluorogenic bands of activity were detected using a Fluor-S™ MultiImager (Bio-RAD). The native molecular weight of protein was estimated by using Sephacryl S-300 (Amersham Phamacia) gel filtration chromatography. The Sephacryl S-300 column (1.5 cm × 25cm, 150 cm^3) was run in 50 mM Tris-HCl, pH 7.0 containing 0.3 M NaCl and calibrated with β-amylase (200 kDa), alcohol dehydrogenase (150 kDal), carbonic anhydrase (29 kDa), cytochrome C (12.4 kDa) as standards.

The N-terminus of the dalcochinase protein purified from *P. pastoris* culture media was sequenced with an ABI 471A Peptide Sequencer (Applied BioSystems Inc., CA, USA). The protein was dried onto a glass fiber filter and sequenced by the standard method.

2.12 β-Glucosidase pH and temperature optimum

To determine the pH optimum for enzyme activity, the reactions were performed in different 0.1 M buffers from pH 3-8.5 (citrate, pH 3-4; NaOAc, pH 4.5-5.5; potassium phosphate, pH 6-8.5), at 0.5 pH unit intervals. The activity at various pH values was

measured by mixing the enzyme solution with 1 mM final concentration of *p*NP-β-D-glucoside or *p*NP-β-D-fucoside. The temperature optimum was determined by incubating the enzyme with 1 mM final concentration of *p*NP-β-D-glucoside or *p*NP-β-D-fucoside in 0.1 M NaOAc, pH 5.0 and incubating at temperatures ranging of 35-85°C, at 5°C intervals for 10 min.

2.13. HPLC and TLC analysis of hydrolysis products

The reaction products after enzymatic hydrolysis of natural substrates were separated and quantified with an Eclipse XDB-C18 (4.6 mm × 250 mm (5 μm)) reverse phase column on an HP-Series 1100 HPLC (Agilent Corp, Palo Alto, CA, USA) with a linear gradient of 0-100% methanol in 0.1% TFA/water.

TLC of hydrolyzed products was performed on analytical silica gel 60 F_{254} aluminum (Merck, Darmstadt, Germany) with $CHCl_3$/MeOH/H_2O (15:3:1) as solvent.

2.14. Production of Gateway Destination vectors from pET32a, pPICZαBNH and pPICZαBNH8.

The Gateway Conversion cassette A (Invitrogen) was blunt-end ligated into the *EcoR*V site of pET32a (+) (Novagen, Madison, WI) according to the Invitrogen Gateway Conversion Kit directions, to create the pET32a (+)/DEST Gateway expression vector. The Gateway Conversion cassette C (Invitrogen) was inserted into pPICZαBNH and pPICZαBNH at the SnaB1 site created by the inserted primers in the same manner. All plasmids were thoroughly sequenced around the insertion to confirm the proper sequence and reading frame had been achieved.

2.15.Cloning of Os4BGlu12 into pBAD/DEST, pMal/DEST, pET32/DEST and pPICZαBNH8/DEST and expression.

The cDNA encoding the mature protein of rice Os4bglu12 β-glucosidase was cloned by RT-PCR and inserted into pENTR-D/TOPO Gateway entry vector and transferred to the pET32a (+)/DEST Gateway expression vector for expression. The cDNA encoding the mature protein of the Os4bglu12 was PCR amplified using cDNA cloned as the template with the Os4bglu12matNcoIf (5'-CACCATGGCCTACAATAGCGCCGGCGAG-3') and Os4bglu12stopr (5'-ATCATTTCAGGAGGAACTTCTTG-3') primers and *Pfu* DNA polymerase to introduce a directional cloning site at the 5' end. The amplification was done as above, but with 45°C annealing temperature. The PCR product was cloned into the pENTR-D/TOPO Gateway entry vector, according to the supplier's directions (Invitrogen).

The cDNA insert in the pENTR-D/TOPO vectors was subcloned into the pBAD/DEST, pET32a (+)/DEST, pMal/DEST, and pPICZαBNH8/DEST Gateway expression vectors by LR Clonase recombination by the recommended protocol (Invitrogen) and sequenced completely. The recombinant pET32a (+)/DEST-*Os4bglu12* plasmid was transformed into *Origami* (DE3) and *OrigamiB* (DE3) strains of *E. coli* by the $CaCl_2$ method (Maniatis et al., 1982), and positive clones were selected on 15 μg/mL kanamycin, 12.5 μg/mL tetracycline and 100 μg/mL ampicillin LB-agar plates, while pBAD/DEST-*Os4bglu12* and pMal/DEST-*Os4bglu12* were transformed into Top 10 *E. coli* by the same method and selected on LB-agar plates with 100 μg/mL ampicillin. The pPICZαBNH8/DEST-*Os4bglu12* plasmid was cloned into *P. pastoris* strain GS115 by electroporation, according to the *Pichia* manual (Invitrogen). For recombinant protein expression, the selected clones were grown in LB medium containing 15 μg/mL kanamycin, 12.5 μg/mL tetracycline and 100 μg/mL ampicillin at 37°C until the optical density at 600 nm reached 0.5-0.6, IPTG was added to a final concentration of 0.3 mM, and the cultures were incubated at 20°C for 8 h. Induced cultures were harvested by centrifugation at 5000×g at 4°C for 10 min. The cell pellets were resuspended in freshly prepared extraction buffer (50 mM phosphate buffer (pH 8.0), 200 μg/mL lysozyme, 1% Triton-X 100, 1 mM phenylmethylsulfonylfluoride, 40 μg/mL DNase I), and incubated at room temperature for 30 min. The soluble protein was recovered by centrifugation at 12,000x g at 4°C for 10 min. The expressed thioredoxin-Os4bglu12 fusion protein was purified by immobilized metal affinity chromatography (IMAC) with TALON cobalt resin according to the manufacturer's instructions (Clonetech, Palo Alto, CA). The fractions with *p*NPG hydrolysis activity were pooled and concentrated with 10 kDa-cut-off centrifugal ultrafiltration membranes (YM-10, Amicon).

2.16. Cloning of human GalA into pPICZαBNH8/DEST and expression

The cDNA from the proband and a normal control were PCR amplified with the primers BamHI-GLA (5′-CACCGGATCCCTGGACAATGGATTGGCAAG-3′) and HindIII-GLA (5′-CCCAAGCTTAAAGTAAGTCTTTTAATGACATCTG-3′), gel purified, and cloned into pENTR/D-TOPO (Invitrogen, Carlsbad, CA, USA). The cDNA were recombined with LR Clonase (Invitrogen) into the *Pichia pastoris* expression vector, pPICZα-BNH8/DEST. The plasmids were transformed into *P. pastoris* strain GS115, and selected with zeocin. Cultures of colonies containing the empty expression vectors, vectors with wildtype GalA or L106R GalA cDNA, were induced to produce protein with 0.5% methanol

according to the *Pichia* manual (http://www.invitrogen.com). Five day media from 3 normal cDNA and 8 mutant cDNA clones were assayed for α-galactosidase A activity.

3. Results

3.1. Plasmids for tagged expression and expression of *Dalbergia cochinchinensis* β-glucosidase.

After unsuccessfully trying to produce *D. cochinchinensis* (Thai rosewood) β-glucosidase (dalcochinase) in several *Escherichia coli* and *Sacchromycees cerevisiae* expression systems, we tried to express it in the pPICZα-Thrombin plasmid provided by Mariena Ketudat-Cairns' group, which is designed to introduce a thrombin site, an S-tag and a His_6 tag at the C-terminus of the proteins. Though the protein could be expressed in active form with high β-glucosidase and β-fucosidase activity, as previously seen in her group, the protein could not be bound to the immobilized metal affinity column (IMAC) with either Ni or Co bound. Therefore, we suspected proteolytic cleavage occurred between the C-terminal region of the protein and the tag. We therefore eliminated a dibasic site, which is frequently a site of proteolysis, by mutating Arg518 of the mature dalcochinase to Trp, which was the sequence deduced from the initial *D. nigrescens* β-glucosidase cDNA clone. However, this failed to resolve the problem of the protein not binding to the resin. Therefore, we undertook two approaches: attachment of the tag to the N-terminus of the protein and further deletion of the C-terminus of the protein. The former approach proved to be more successful.

In order to put an N-terminal His-tag onto the protein for expression, we incorporated it in the pPICZα-thrombin plasmid by introducing a short synthetic DNA between the *Pst* I site and the EcoRI site of the vector, which added a His-tag, eliminated the initial *Pst*I site, replaced the *Pst*I site, and inserted a *Sna*BI site between the *Pst*I site and the *Eco*RI site. This strategy was meant to incorporate the His-tag and return the multiple cloning site (MCS) in to the original reading frame to allow easy transfer of inserts from previous pPICZα constructs. The addition of the *Sna*BI site allowed for an easy check on whether the synthetic DNA had been inserted and provided a blunt-end restriction site for later insertion of a Gateway conversion cassette to convert the expression plasmids to the destination vectors for cDNA in cloned into the Gateway system. The inserts were found to be incorporated in all clones checked by *Sna*BI insertion and sequencing. The initial plasmid, pPICZαBNH, contained a His_6 tag, followed by an enterokinase (EK) site for facile removal of the tag after expression. However, this plasmid again produced protein with high β-fucosidase activity (more diagnostic of success in expression than β-glucosidase due to endogenous *Pichia* β-glucosidase activity, which is also induced by methanol, unpublished results and Xu et al., 2006) that was not bound to the IMAC column. Since this might be due to cleavage within

the enterokinase site or poor binding of the His-tag, another construct, pPICZαBNH8, was produced with the same strategy, but with a longer His tag of 8 residues and with no EK site. Again, insertion of the dalcochinase cDNA into this vector resulted in secretion of active protein that was not bound by IMAC resin into the media.

Because there seemed to still be a problem with the His-tag, we investigated where the N-terminus of the protein was. Protein obtained by fermentation of *P. pastoris* producing dalcochinase from the pPICZα plasmid, which had been purified from the media by expanded bed chromatography on Streamline Direct HST2 resin (Charoenrat et al., 2006), followed by gel filtration chromatography was submitted for automated Edman sequencing by Dr. Chantragan Srisomsap at Chulabhorn Research Institute. The sequence obtained was EVPPFN, which indicated that the first 12 amino acids of the dalcochinase had been cleaved from the protein, along with any N-terminal tag in the *Pichia pastoris* system (Figure 1).

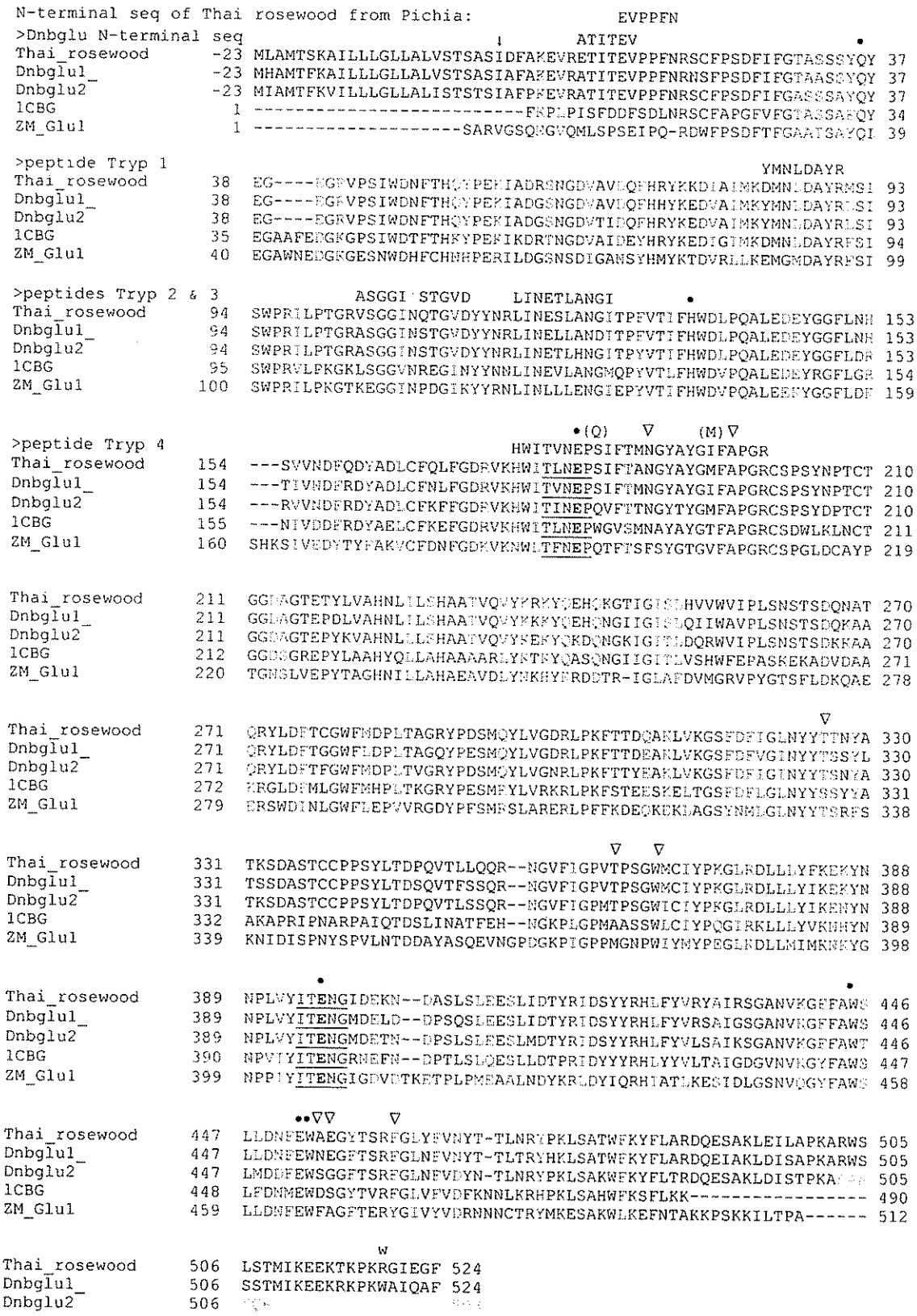

Figure 1: Alignment of the sequences of Thai rosewood dalcochinase, the *D. nigrescens* bglu1 and bglu2 cDNA-derived proteins and white clover linamarase (1CBG) and maize Glu1 (ZM_Glu1). The peptides determined by Edman degradation protein sequencing are shown above the aligned sequences. A small arrow (↓) markes the amino terminus of dalcochinase from seed, The catalytic acid/base and catalytic nucleophile are in bold with their motif's underlined. Other residues in the glycone binding site are marked by dots (•), while aglycone binding residues are marked by triangles (∇). Successful expression constructs started from V14, the second residue in the N-terminal sequence of dalcochinase from *Pichia* media. Dalcochinase residue R519, which was mutated to W to eliminate the dibasic is marked with w.

In order to produce a new protein without the apparent protease site in the N-terminus of the dalcochinase protein, a new primer was designed to amplify the cDNA encoding the protein starting from the sequence VPPFN with a *Pst*I site in front for cloning into the pPICZαBNH8 plasmid. This primer, along with a C-terminal primer including the stop codon, was used to amplify the cDNA insert from the previous expression construct and it was inserted into the pPICZαBNH8 expression vector and sequenced to show it had the correct sequence. When the plasmid, designated pPICZα-His8-trncTRBG, was transformed into *Pichia pastoris*, the protein could be bound to IMAC resin from desalted media, though some remained in solution. Alternatively, the protein was first partially purified by hydrophobic interaction chromatography (Toonkool et al., 2006), but with simlar effects. A final yield of about 44% compared to activity in the media was obtained by the latter procedure, and the specific acitivity of this protein was approx. 14 units/mg (Table 3). When a small amount of desalted media was bound to a Ni IMAC column, only 35% of the β-glucosidase activity was recovered in the elution frations, but when it was bound to a Co-containing IMAC column, 75% was recovered in the elution fractions. However, loading of larger amounts of protein onto the column appeared to result in rapid saturation of the resin with further protein eluting in the flow-through. Therefore, the use of the N-terminally truncated dalcochinase protein linked to the N-terminal His-tag allowed purification of the protein by IMAC, though the procedure was not so efficient for large amounts of protein.

Table 3: Purification of recombinant dalcochinase from culture medium of *P. pastoris* containing the pPICZα-His$_8$-trncTRBG. One liter of culture was used for purification, and assays were performed with 1 mM *p*NP-Glc (Toonkool et al., 2006).

Fraction	*Total activity (unit)*	*Total protein (mg)*	*Specific activity (unit/mg)*	*Purification (fold)*	*Yield (%)*
Culture medium	55.0	92.8	0.6	1.0	100
Phenyl sepharose	38.8	32.8	1.2	2.0	71
Ni^{2+} affinity	24.4	2.0	12.5	21.1	45
Ultrafiltration	24.1	1.8	13.8	23.3	44

We also tried to construct the protein with a C-terminal His-tag by truncating the C-terminus at the position of the last amino acid residue in the 1CBG structure of white clover linamarinase (Barrett et al., 1995). When the cDNA encoding the protein that ended at Arg488 (R488) was cloned into pPICZαB in frame with the C-terminal cmyc and His-tags, very little activity could be found in protein secreted from *P. pastoris* clones harboring this

construct, and β-fucosidase activity was lower than β-glucosidase activity, suggesting the protein may not be very active and *P. pastoris* β-glucosidase was responsible for a significant amount of the activity. However, when this media was passed over IMAC, two bands of around 66 kDa could be seen on SDS-PAGE. So, it seems likely the construct eliminated a protease site, but it also destroyed the activity of the protein.

The N-terminally truncated protein was kinetically characterized. The protein was found to have a pH optimum of 5.0 and a temperature optimum of 60° C in the standard 10 min assay, similar to the protein purified from seed (Srisomsap et al., 1995). The protein also had kinetic constants for hydrolysis of *p*NP-β-D-glucoside and *p*NP-β-D-fucoside similar to the protein purified from seed, as shown in Table 4. Therefore, we were able to successfully produce dalcochinase with near native enzymatic properties in *P. pastoris* in a readily purifiable form.

Table 4: Comparison of Michaelis-Menton parameters for hydrolysis of *p*NP glycosides of Thai rosewood dalcochinases purified from seed and purified from recombinant *Pichia pastoris* media.

Enzyme:	Dalcochinase from Seeds*		Dalcochinase from Recombinant *Pichia pastoris*	
Substrate	*p*NP-Glc	*p*NP-Fuc	*p*NP-Glc	*p*NP-Fuc
K_m (mM)	5.37 +/- 0.09	0.54 +/- 0.04	5.07 +/- 0.07	0.55 +/- 0.02
k_{cat} (s^{-1})	307 +/- 5	151 +/- 3	206 +/- 7	156 +/- 22
k_{cat}/K_m ($M\ s^{-1}$)	57,300	283,100	40,600	282,000

*p*NP-Glc = *p*-nitrophenyl-β-D-glucopyranoside, pNP-Fuc = *p*-nitropheyl-β-D-fucopyranoside
* The values for dalcochinase from seed are taken from Srisomsap et al., 1995.

3.2 *Dalbergia nigrescens* β-glucosidase

In a previous grant, we had purified the β-glucosidase from *D. nigrescens* and cloned a cDNA from the seeds. The protein from *D. nigrescens* seeds hydrolyzed pNP-Glc and *p*NP-Fuc somewhat less efficiently than dalcochinase, but had the interesting property of efficiently hydrolyzing isoflavonoid 7-O-diglycosides, in addition to isoflavone 7-O-β-D-glucosides (Chuankhayan et al., 2005). However, attempts at expression of this protein in *P. pastoris* and *E. coli* from the cloned cDNA resulted in no significant β-glucosidase/β-

fucosidase activity. It was noted that Asp127 corresponded to a conserved Gly in other plant GH family 1 β-glucosidases, so this residue was mutated to glycine, but this mutant cDNA also failed to produce active protein. A stop codon was previously found in place of the conserved Trp95 codon, so this segment of the cDNA was reamplified from fresh *D. nigrescens* seeds. When this cDNA fragment was cloned and sequenced, it was found to have a slightly different sequence, so specific primers were designed from it for 3' and 5' RACE, which was used to amplify the rest of the *dnbglu2* cDNA, which encodes the dnbglu2 protein in Figure 1. The full-length coding region dnbglu2 cDNA was amplified with specific primers designed from the RACE product.

The full-length *Dnbglu2* cDNA sequence consisted of 1,964 nucleotides, which included a 1,593-nucleotide ORF encoding a 531 amino acid precursor protein, Dnbglu2 (Figure 1). The Dnbglu2 protein was predicted to contain a 23 amino acid signal sequence by the SignalP program (Benetsen et al., 2005), which leaves a 508 amino acid mature protein with a calculated molecular mass of 60,509 Da. The predicted N-terminal sequence (I-A-F-P-K) corresponded to the mature N-terminus of *D. cochinchinensis* β-glucosidase (I-D-F-A-K) [13] (Figure 1). However, N-terminal sequencing of Dnbglu purified from seeds previously showed the sequence A-T-I-T-E-V (Chuankhayan et al., 2005), which occurs 8 residues later than the predicted sequence, possibly due to proteolysis after cleavage of the signal sequence, either in the plant or during purification. Using the N-terminal sequence determined by Edman degradation as the starting point, the predicted mature mass of Dnbglu2 is 59,571 Da. The shorter length of the predicted Dnbglu2 protein compared to dalcochinase, seen in Figure 1, was due to a frame-shift in the C-terminal coding region at codon 526, which resulted in a stop codon 5 amino acids later. This sequence was confirmed by repeated sequencing.

As shown in Figure 1, the predicted Dnbglu2 protein sequence is slightly different from the four peptide sequences previously determined from β-glucosidase purified from *D. nigrescens* seeds (Chuankhayan et al., 2005). As noted, the chemically sequenced N-terminus matched the Dnbglu2 sequence, but the predicted N-terminus preceded it by 8 residues. The Dnbglu2 sequence also matched that of tryptic peptide 1. With tryptic peptide 2, all residues matched, except that residue 6 showed absence of the expected Asn signal, and instead showed low levels of the preceding Ile residue, suggesting that residue 6 is likely to be glycosylated. The Dnbglu2 sequence showed one mismatch with tryptic peptide 3, at the 7^{th} residue. Tryptic peptide 4 matched the sequence of Dnbglu2 at 19 out of 25 residues, with some minor signals matching the Dnbglu2 sequence for some residues where the primary signal was different. So, the purified Dnbglu might contain more than one isozyme with

similar peptides eluting together in the HPLC tryptic map. It is also possible that some mutations could have occurred due to mutations during reverse-transcription and amplification, though the use of a high fidelity polymerase for amplification should have minimized this, so it could not have accounted for all the differences seen in the protein and DNA-derived sequences.

When the predicted protein sequence was compared with the sequences of isoflavonoid β-glucosidases and other closely related β-glucosidases, Dnbglu2 was most closely related to the predicted protein from *D. nigrescens* Dnbglu2 and the Thai rosewood dalcochinase, as shown in the phylogenetic tree in Figure 2. The protein had 82% amino acid sequence identity with Dnbglu1 and 81% identity with dalcochinase, and these enzymes grouped in a cluster that was somewhat more distant from other legume β-glucosidases, which were more closely related to the cyanogenic β-glucosidases from cherry and white clover. Interestingly, while *D. nigrescens* β-glucosidase can hydrolyze isoflavone 7-O-diglycosides (E.C. 3.2.1.161, Chuankhayan et al., 2005), the other diglycosidases included, furcatin hydrolase and primeverosidase, were grouped in a more distantly related cluster.

When recombinant *Dnblu2* starting from the predicted N-terminus was expressed in fusion with yeast alpha-factor prepropeptide from pPICZα-thrombin in *P. pastoris*, increased *p*NP-Fuc hydrolase activity appeared in the media, but the protein could not be purified from by IMAC, similar to the problem with *D. cochinchinensis* dalcochinase. The protein could be partially purified by gel filtration, and, by comparison of the elution volume to that of molecular weight standards, the native molecular weight of recombinant Dnbglu2 β-glucosidase was estimated to be about 240 kDa by S-200 gel filtration chromatography, which suggests that this recombinant protein is composed of 4 subunits, as are the native β-glucosidases purified from *D. cochinchinensis* and *D. nigrescens* seeds (Srisomsap et al., 1995, Chuankhayan et al., 2005). To develop a facile purification system, the cDNA was cloned into the pPICZαBNH and pPICZαBNH8 plasmids, but the experience was similar to that of dalcochinase and no protein could be purified from the media by IMAC. Finally, the cDNA encoding the protein starting from the VPPFN sequence, which is conserved with dalcochinase, was amplified and cloned into the pPICZαBNH8 plasmid. When this construct was used to produce active protein in *P. pastoris*, an approximately 63 kDa protein could be purified from desalted media by adsorption to cobalt chelating resin (IMAC), as shown in Figure 3. So, the same strategy that worked with Thai rosewood dalcochinse also worked to produce readily purifiable Dnbglu2 β-glucosidase.

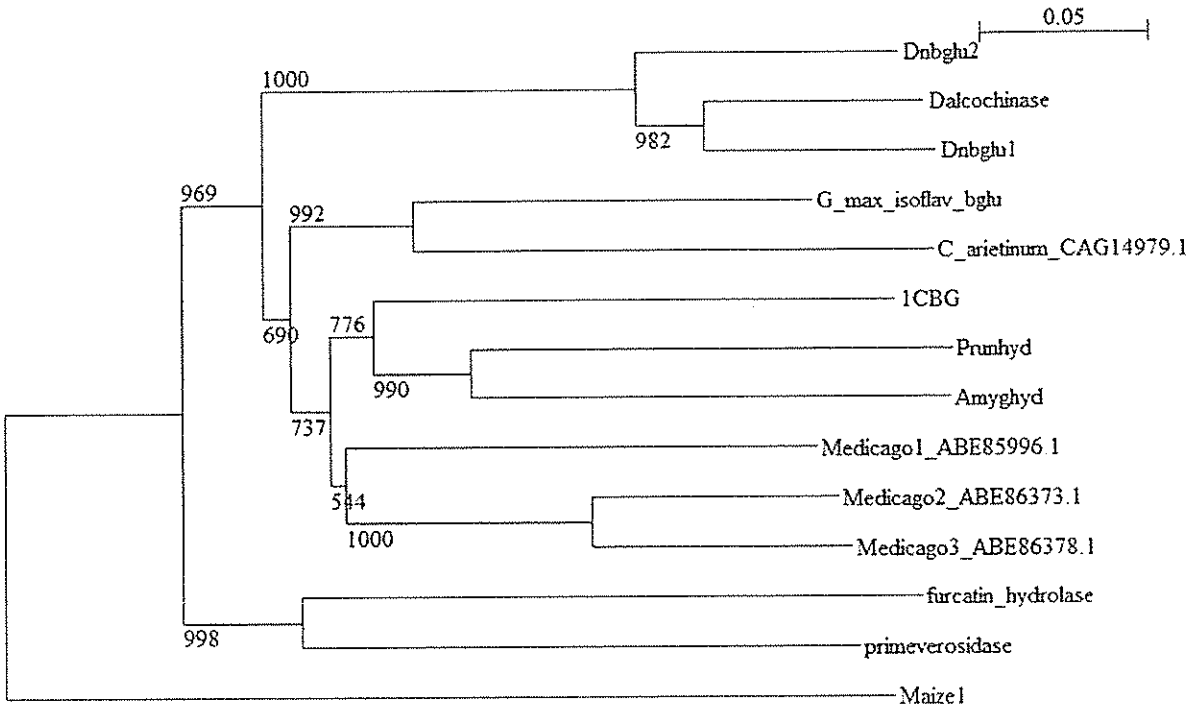

Figure 2: Phylogenetic tree of *Dalbergia* isoflavonoid β-glucosidases with legume β-glucosidases and disaccharidases. The Maize Glu1 β-glucosidase (Maize1, accession no. **AAB03266**) is included as an outgroup. The tree shows that the *Dalbergia* isoflavonoid β-glucosidases (*D. nigrescens* Dnbglu1 and Dnbglu2 and *D. cochinchinensis* dalcochinase, Dcbglu) group together, while the soybean isoflavonoid conjugate-specific β-glucosidase (G_max_isoflav_bglu, Suzuki et al., 2006; accession no. **BAF34333.1**) groups with chickpea β-glucosidase (C_arietinum, accession no. **CAG14979**) in another cluster. These enzymes are closely related to cyanogenic β-glucosidases from clover (1CBG, Barrett et al., 1995) and cherry (*Prunus serotina* Prunhyd, prunacin hydrolase, accession number AAL07435; Amyghyd, amygdalin hydrolase, accession no. **AAL07489**) than they are to furcatin hydrolase (*Viburnum furcatum*, accession no. **BAD14925**), despite the latter showing more similar substrate specificity (Ahn et al., 2004). Also included are *Camellia sinensis* β-primeverosidase (accession no. **BAC78656**) and three protein sequences derived from *Medicago trucatula* genes (Medicago1, 2 and 3), which include accession numbers in their names. This tree was produced by the neighbor-joining method, and the same branching was attained by the maximum parsimony method. Bootstrap values for reproducibility out of 1000 trials are given at the internal tree branches.

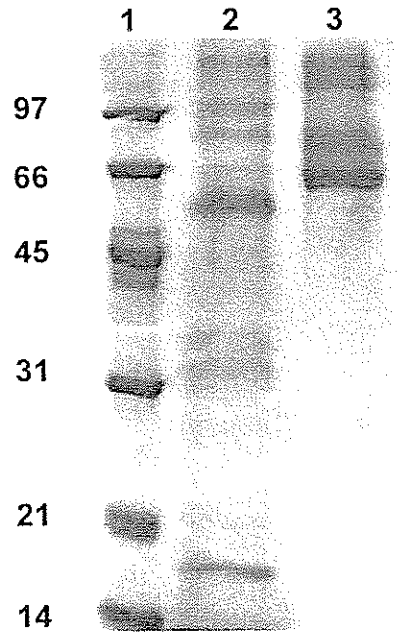

Figure 3. SDS-PAGE analysis of *Dalbergia nigrescens* Dnbglu2 β-glucosidase purified from *Pichia pastoris* media. Lane 1, Low MW markers with molecular weights shown to the left; lane 2, Crude desalted media; lane 3, IMAC purified Dnbglu2 β-glucosidase. Note that the main bands were two closely spaced genes around 64 kDa, though higher MW bands, some of which are likely hyperglycosylated forms and undispersed aggregates of the Dnbglu2 protein.

The relative hydrolysis rates of the recombinant *Dalbergia* β-glucosidases toward *p*NP-glycoside substrates were determined. Relative hydrolysis rates of the enzymes expressed in *Pichia* were similar to those for enzymes purified from seeds, but Dnbglu2 gave slightly higher relative activity toward β-D-fucoside and β-D-xyloside and could not detectably hydrolyze β-D-mannoside, α-L-arabinoside, and β-D-thioglucoside (Table 5). The recombinant dalcochinase gave lower relative activity for *p*NP-β-D-galactoside, but showed higher relative activity towards *p*NP-β-D-mannoside compared to dalcochinase from seeds. Dnbglu2 showed similar K_m values for both *p*NP-Glu (26 +/- 3 mM) and *p*NP-Fuc (1.67 +/- 0.08) as natural *D. nigrescens* β-glucosidase from seeds (Chuankhayan et al., 2005, 2007b). The recombinant *Dalbergia* β-glucosidases were also compared for their hydrolysis of isoflavonoid glycosides, including their own natural substrates, as shown in Table 6 and Figure 4.

Table 5 Hydrolysis of glycoside substrates with recombinant *D. nigrescens* β-glucosidase compared with *D. nigrescens* β-glucosidase purified from seed and *D. cochinchinensis* β-glucosidase. Assays were done with 5 mM substrate for 10 min under standard assay conditions.

Substrates	% Relative activity			
	Natural Dnbglu	Dnbglu2	Natural *D. cochinchinensis*	Wild type *D. cochinchinensis*
*p*NP-β-D-glucoside	100	100	100	100
*p*NP-β-D-fucoside	124	139	124	127
*p*NP-β-D-galactoside	3.97	4.4	8.95	4.65
*p*NP-β-D-xyloside	7.55	12.8	3.91	2.95
*p*NP-α-L-arabinoside	1.91	nd	4.89	3.74
*p*NP-β-D-thioglucoside	0.68	nd	0.02	-
*p*NP-β-L-arabinoside	0.47	1.8	-	-
*p*NP-β-D-mannoside	0.4	nd	0.26	5.91

nd: not detectable; - not determined

Table 6 Hydrolysis of isoflavonoid glycosides by Dnglu2 relative to recombinant dalcochinase

Enzyme	Isoflavonoid Substrate				
	I	II	III	Daidzin (VI)	Genistin (V)
Dalcochinase	1.0	1.0	1.0	1.0	1.0
Dnbglu2	0.00031	290	210	3.1	2.8

Assays were done with 1 mM substrates for 10 min under standard conditions and release of *D. nigrescens* isoflavonoids from II (dalpatein-7-O-β-D-apiofuranosyl-1,6-β-D-glucopyranoside) and III (dalnigrein-7-O-β-D-apiofuranosyl-1,6-β-D-glucopyranoside) was quantified by HPLC, while glucose release from I (dalcochinin β-D-glucoside), VI (diadzin) and V (genistin) was quantified by glucose oxidase assay. The specific activities of Dnbglu2 from *D. nigrescens* are given relative to wildtype *D. cochinchinensis* dalcochinase.

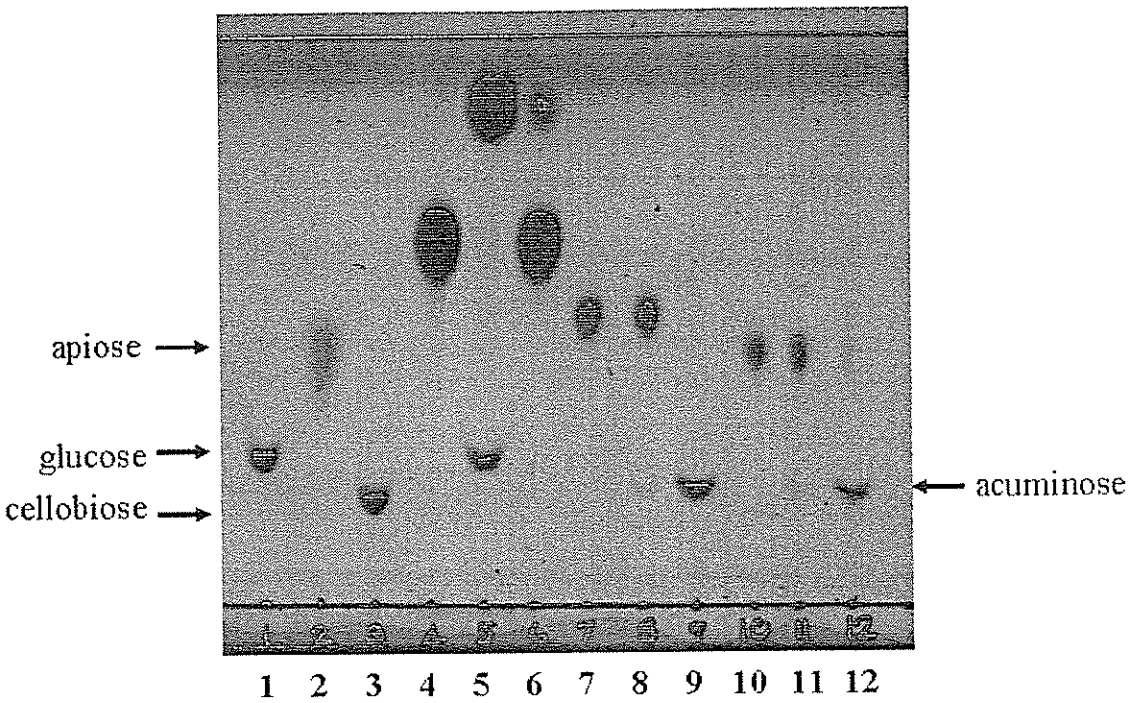

Figure 4. TLC of I, II and III hydrolysis by recombinant *D. nigrescens* Dnbglu2 and *D. cochinchinensis* dalcochinase. Digests were done with 0.01 unit for 16 hrs at 30°C. Lane 1, glucose std; lane 2, apiose std; lane 3, cellobiose std; lane 4, I std; lane 5, I hydrolyzed with recombinant *D. cochinchinensis*; lane 6, I hydrolyzed with Dnbglu2; lane 7, II std; lane 8, II hydrolyzed with recombinant dalcochinase; lane 9, II hydrolyzed with Dnbglu2; lane 10, III std; lane 11, III hydrolyzed with recombinant *D. cochinchinensis*; lane 12, III hydrolyzed with Dnbglu2. For definitions of I, II, and III, see Table 6.

3.3. Development of High throughput Expression Screening System.

In order to facilitate the work on several projects that require the testing of different expression systems, we developed a Gateway® screening system to include plasmids for different types of bacterial expression in *E. coli* and expression as secreted proteins in *P. pastoris*. In the Gateway system, clones are introducted into entry vectors, from which they can then be transferred to any number of destination vectors for expression. Though we could purchase or borrow some destination expression vectors, some of the most productive vectors in our laboratory did not have a corresponding destination vector, so we had to convert them to the system.

For entry vectors, we have used 4 different kinds from the company. First, we acquired pENTR4, which can be reproduced in the laboratory and allows cloning of inserts by restriction digestion. This is convenient when the cDNA can be cloned with the appropriate restriction sites at the end and these restriction sites are not found within the cDNA clone, but

this still takes time. To eliminate any need for the lack of restriction sites within the clone, we bought the pENTR-D/TOPO and pENTR-TEV-D/TOPO plasmids, which allowed cloning of blunt end PCR fragments directly by a topoisomerase reaction. In order to allow convenient transfer and selection into vectors with kanamycin resistance, we ordered the pDONR-ZEO vector. This vector allows cDNA to be cloned into it by the BP clonase reaction between PCR products or other clones that contain B recombination sites at the ends (such as most of the DEST expression constructs), and has a zeocin site to allow selection that is different from destination vectors that have kanamycin resistance, such as the pMDC plant expression vectors, which we aquired from the University of Zurich (Curtis and Grossniklaus, 2003). The pENTR-TEV-D/TOPO plasmid has the advantage of providing a TEV protease site after the recombinantion site, allowing the tag, including the cloning site to be removed from the recombinant protein after production by proteolysis. However, the addition of the TEV site may affect the solubility of the recombinant protein, so it was is advantageous to try cloning the plasmids with both the pENTR-D/TOPO plasmids. Several cDNA encoding mature proteins were cloned with each of these plasmids for transfer to expression vectors, as part of our project to characterize express rice glycosyl hydrolase family1 and family 35 proteins.

To provide the expression vectors for producing the proteins from the Gateway system, we acquired the pBAD/DEST vector from Invitrogen, and the pMalc/DEST, pMals/DEST, and pThio/DEST vectors from the Structural Biology group of the Salk Institute (La Jolla, CA), and produced our own destination vectors from pET32a, pPICZαBNH and pPICZαBNH8. The Gateway conversion cassette A was inserted into the pET32a EcoRV site to create the pET32/DEST vector, while the conversion cassette C was ligated into the SnaB1 sites of pPICZαBNH and pPICZαBNH8 vectors to create pPICZαBNH/DEST and pPICZαBNH8/DEST, respectively. Restiction digests and sequencing identified clones with the correct orientation of the insert and confirmed their sequences. The cDNA for various glycosidases were then inserted into these vectors from the appropriate Entry vector and the resulting entry vectors were used to produce proteins in the appropriate host cells as parts of other projects.

The proteins for which cDNA have been cloned into the Gateway system to successfully produce protein include rice Os1BGlu1, Os3BGlu6, Os3BGlu8, Os4BGlu12, Os7BGlu26, Os9BGlu31, OsBgal3, OsBgal4, OsBgal5, and OsBgal13, barley BGQ60 β-glucosidase/β-mannosidase, and human GalA α-galactosidases. Most of these proteins were

produced from the pET32/DEST vector in *E coli* strain Origami (DE3) or OrigamiB (DE3), but Os4BGlu12 and OsBgal13 were produced in both *E. coli* and *P. pastoris* (from pPICZαBNH8/DEST), while human GalA α-galacosidase and its mutant were only produced in *P. pastoris*.

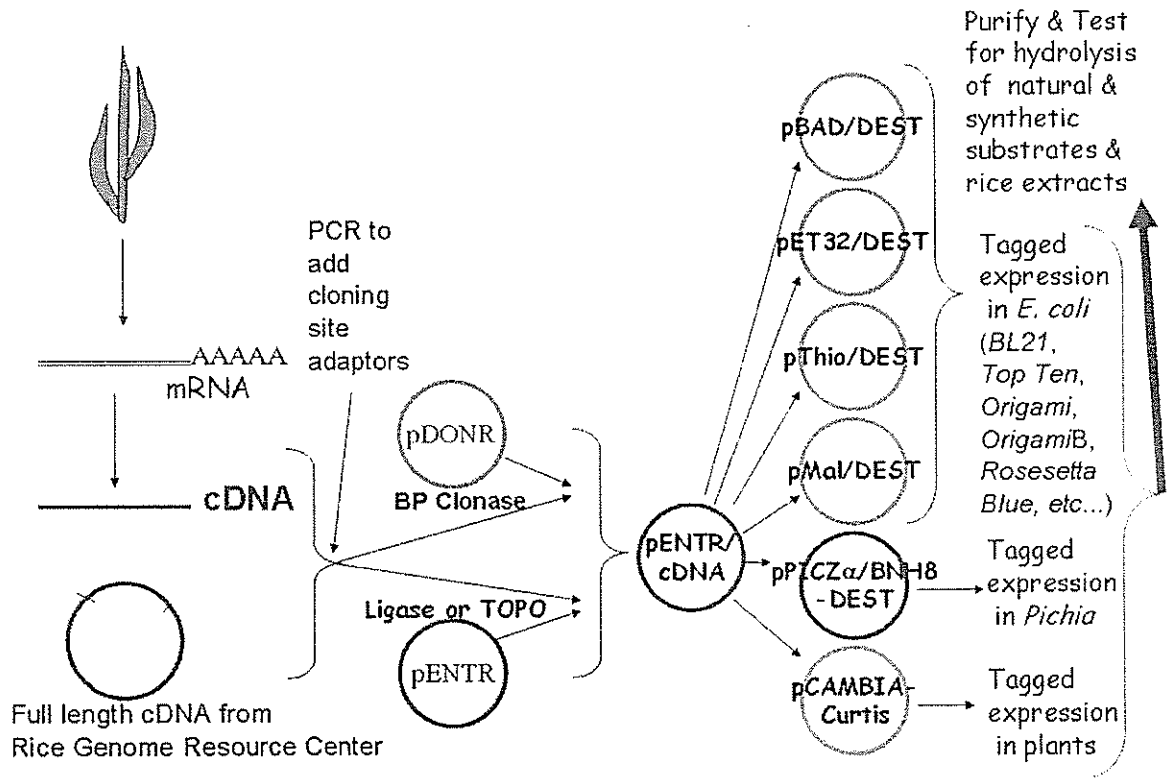

Figure 5. Gateway expression system set-up for expression of recombinant proteins. The diagram shows the system set-up for quickly testing recombinant expression proteins by cloning into an entry vector by PCR amplification of the cDNA encoding the protein for expression, followed by recombinantion into pDONR (pDONR/ZEO from Invitrogen) or ligation into the appropriate sites in pENTR4 or topoisomerase cloning into pENTR-D/TOPO or pENTR. The entry clone produced by this process could then be recombined into a number of vectors for expression in *E. coli*, *P. pastoris* or plants by an LR clonase reaction. The expression in plants was different in two respects, first the pCAMBIA-derived expression vectors are selected on kanamycin in bacteria, so they were not compatible with the pENTR vectors, which also have kanamycin selection, and had to use entry clones derived from pDONR/Zeo, and second, the cDNA required their own signal sequences for targeting to secretion, which were not desireable for the *E. coli* and *P. pastoris* expression systems.

To test the system, the original clone tested was Os4bglu12, the cDNA of which was cloned into the pENTR-D/TOPO vector and then transferred into the pBAD/DEST, pMalc/DEST, pMalp/DEST, pET32/DEST, and pPICZαBNH8/DEST vectors by LR clonase recombination, and tested for expression in various cells. Expression in pBAD/DEST was

first tested with the recommended Top 10 cells and with optimization of the amount of arabinose used to induce the *ara*-BAD promoter, from which the protein is expressed in this system, along with the time and the temperature. As shown in Table 7, the optimal conditions were 0.4% arabinose induction for 8 h at 20°C. Though the activity in the media in this system appeared reasonably good, when the pET32/DEST construct was tested in either Origami (DE3) or OrigamiB (DE3), the expression levels were much higher (Table 3). When the clones of the pMalc/DEST and pMals/DEST with *Os4bglu12* for expression of the protein as N-terminal maltose-binding protein fusionis in the *E. coli* cytoplasm or periplasm, respectively, were tested for b-glucosidase activity in cell lysates after induction, no significant activity was detected in any conditions tested, so this system did not seem efficient for production of this protein. On the other hand, high amounts of activity were seen when the protein was produced in *P. pastoris* from the pPICZαBNH8/DEST-*Os4bglu12* vector with 0.5% methanol induction. Therefore the system of pET32/DEST-Os4bglu12 in OrigamiB (DE3) cells was chosen for expression in bacteria, while the *Pichia* system also produced large amounts of activity.

Table 7. Specific activity and optimal conditions for production of OsBGlu12 in *E. coli* and *P. pastoris* systems.

Expression System	Maximum activity (μmol/mg protein/min)	Optimal conditions tested
pBAD/DEST in Top 10 cells	0.012	0.4% arabinose, 8 h, 20°C
pET32/DEST in *Origami* cells	1.1	0.3 mM IPTG, 18 h, 20°C
pET32/DEST in *OrigamiB* cells	1.15	0.1 mM IPTG, 16 h, 20°C (large scale for purification)
pMalc/DEST (cytoplasmic)	Not detectable	None found
pMals/DEST (secreted)	Not detectable	None found
pPICZαBNH8/DEST	1.62	0.5% MeOH in BYYM, 3 days, 30°C

The protein produced by the OrigamiB *E. coli* system was purified by IMAC to give asingle band, as shown in Figure 6. This protein was compared to the β-glucosidase in *P. pastoris*, which could also be purified, though with low yield which did not allow a clear band on SDS-PAGE to be visualized, as shown in Figure 7. Nonetheless, some activity could be purified and was further characterized. As shown in Figure 8, a comparison between the relative activities toward different pNP-glycosides between the protein produced in *E. coli* and in P. pastoris, showed that the proteins were quite simlar. There was a somewhat lower β-fucosidase acitivity in the protein produced in *P. pastoris*, which might have occurred due to

contamination with *P. pastoris* β-glucosidase, which would make β-glucosidase activity relatively high compared to β-fucosidase, or could be due to real protein differences due to differences in posttranslational modifications, like glycosylation.

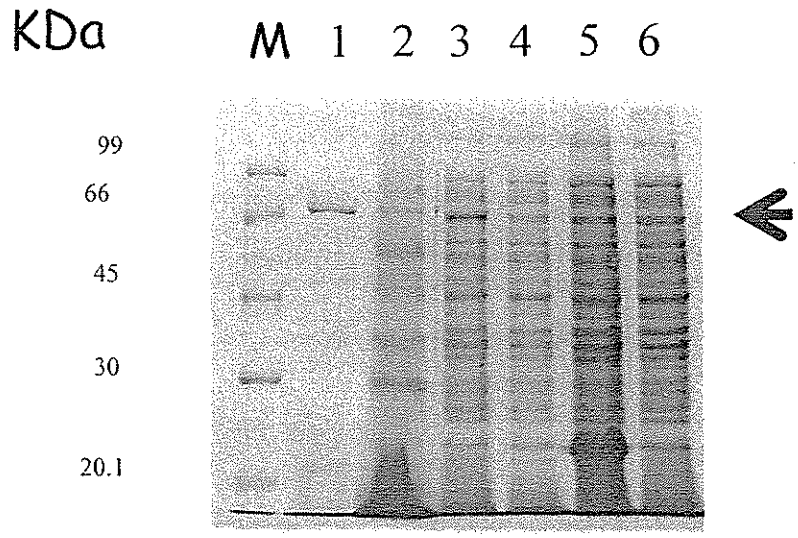

Figure 6. SDS-PAGE analysis of Os4BGlu12-thioredoxin fusion protein produced from pET32/DEST-Os4bglu12 in *OrigamiB* (DE3) *E. coli*. Lane M, low MW markers; lane 1 IMAC purified Os4BGlu12-thioredoxin fusion protein from this system; lane 2 soluble extract of OrigamiB (DE3) *E. coli* cells with pET32/DEST-Os4bglu12 after 16 h induction; lane 3, insoluble fraction of cells with pET32/DEST-Osbglu12 after 16 h; lane 4 insoluble fraction of these cells after 0 h induction; lane 5, whole cell extract of cells with control pET32 plasmid after 16 h induction; and lane 6, whole cell extract of control cells without induction. All cells were grown at 37 C, then induced at 20C.

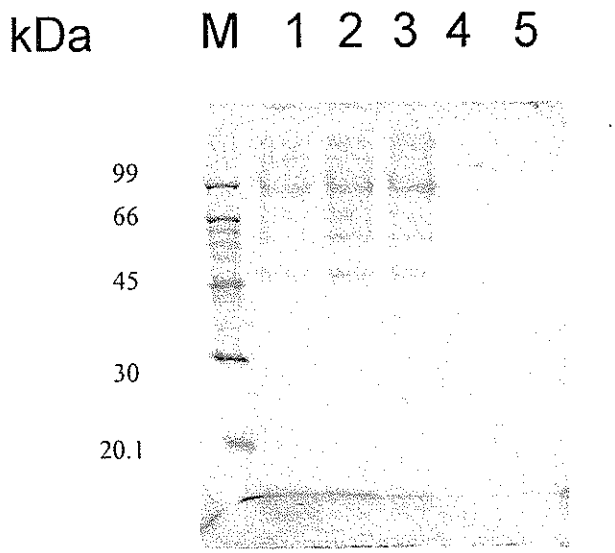

Figure 7. SDS-PAGE analysis of OsBGlu12 production in *Pichia pastoris*. Lane M, Low MW markers; lanes 1-3, media from *P. pastoris* induced to produce OsBGlu12 after 1-3 days, respectively; lane 4 IMAC purified Os12BGal12 from *Pichia* media; lane 5 IMAC elution fractions from nonrecombinant *P. pastoris* media.

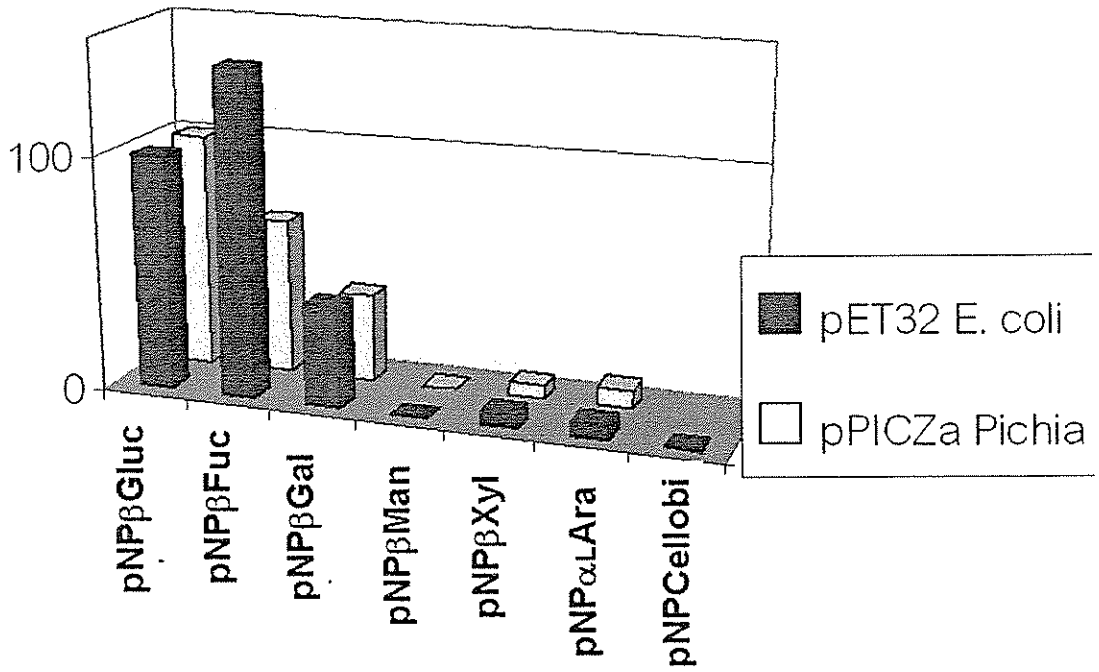

Figure 8. Relative activities toward different pNP-glycosides of Os4BGlu12 produced in *E. coli* and *P. pastoris*. The front row shows the relative activities of the enzyme produced in *E. coli*, while the back row shows the relative activities of the protein produced in *P. pastoris*. All pNP-glycoside substrates were assayed at 1 mM concentration at 30°C, in 50 mM sodium acetate, pH 5.0, for 10 min. The activities are are given relative to the activity toward *p*NP-β-D-glucoside (*p*NPβGluc), which was set at 100%. The other glycosides are *p*NP-β-D-fucoside (*p*NPβFuc), *p*NP-β-D-galactoside (*p*NPβGal), *p*NP-β-D-mannoside (*p*NPβMan), *p*NP-β-D-xyloside (*p*NPβXyl), *p*NP-α-L-arabinoside (*p*NPαAra), and *p*NP-β-D-cellobioside (*p*NPCellobi).

Based on the experience with expression of Os4BGlu12, other enzymes were primarily tested for expression in *E. coli* from pET32/DEST and in *P. pastoris* from pPICZαBNH8/DEST. Many glycosidases from rice studied in our lab were tested, as listed above and others that were not successful. In general, there is sometimes a problem that the protein is not detected under any conditions in *E. coli*, not even the N-terminal tag, which was fused to the protein. For 8 genes for which the cDNA was cloned into the system, the protein of which could not be expressed in *E. coli*, no activity could be detected when they were expressed in *P. pastoris*, as well. However, the *P. pastoris* system was still useful if the protein could be produced in *E. coli*, but in insoluble inactive form. Close inspection of the gene sequences for the proteins that could not be expressed did not indicate any problems with the coding region (which would have left the N-terminal tag intact, if they had occurred). In addition, the presence of rare codons, which might prevent translation, was not verified by trying to produce some of these proteins in Rosetta (DE3) and Rosettagami (DE3) cells that

have extra tRNA to match the rare codons. Therefore, the reason for lack of expression of proteins from some cDNA remains unclear, but the system could be used to express about half of the genes that were attempted. More work will be done in the future on some of the unexpressable genes of interest to see where the problem occurs.

One additional problem that was addressed with this system was the need for rapid assessment of mutant proteins for their activity. We cloned the *GalA* gene cDNA from a patient with Fabry disease and from a normal control into the pENTR-D/TOPO plasmid and transferred them into pPICZαBNH8/DEST for expression in *P. pastoris*. When mutant and normal cDNA were used to express protein in *P. pastoris*, three normal clones were all found to express >2200 nmoles/h/mg protein of α-galactosidase activity in 5 day media, but of eight mutant clones studied, seven showed no α-galactosidase on 5 day, and one showed much lower activity than normal (39 nmoles/h/mg protein). Thus, the system allowed rapid assessment of the causative nature of the mutation, based on the protein activity.

Section 4 Analysis

4.1 Discussion:

This project had 3 main components: production of expression vectors for tagged expression of *Dalbergia* β-glucosidases and expression of *D. cochinchinensis* dalcochinase in the appropriate system, cloning of *D. nigrescens* Dnbglu2 cDNA and expression of its protein in the *Pichia pastoris* system that was developed, and development of a rapid system for cloning cDNA into various constructs for expression trials. In the first section, initially we tried expression in the pET32a vector in Origami cells, as had been successful for rice BGlu1 β-glucosidase (Opassiri et al, 2003) and *Leucaena leucocephala* chitinase (Kaomek et al., 2003), and in the pET39 and pET40 vectors, which allow secretion in *E. coli*. However, only insoluble and inactive protein was produced for both *D. cochinchinensis* and *D. nigrescens* b-glucosidases in these systems, so we returned to the *P. pastoris* system that had previously been successful for expression of the *D. cochinchinesis* dalcochinase (Ketudat Cairns et al., 2000) and introduced a polyhistidine tag for affinity purification of the protein. Though it took several attempts to introduce a tag that was not removed by proteolysis and left an active protein, we were finally able to express β-glucosidases from both *Dalbergia* species in this manner. However, it was necessary to clone a new cDNA for the *D. nigrescens* β-glucosidase, since the previously cloned cDNA could not produce acitive protein in the *Pichia* system. Finally, we developed the plasmids produced from this work for expression in *P. pastoris* and several *E. coli* expression vectors into an integrated system for rapid transfer of genes between expression vectors with the Gateway System from Invitrogen. This allowed us to work on several other projects to characterize rice β-glycosidase proteins, as well as human α-galactosidase, and puts us in a good position to biochemically characterize the products of genes of interest in the future.

In the part of the work where we tried to find appropriate constructs for expression and IMAC purification of b-glucosidases in Pichia pastoris, several different approaches were tried. Initially, we eliminated a dibasic site to prevent cleavage of the C-terminus, but found the protein still did not bind to IMAC resin. Cutting off the C-terminus to the point of the C-terminus of the 1CBG β-glucosidase structure (Barrett et al., 1995) appeared to allow at least some of the protein to bind to the IMAC column, but very little activity was seen, suggesting that this construct affected the protein's activity. Initially, moving the His-tag to the N-terminus seemed to not be productive, as well. Lengthening of the histidine tag to 8 residues and removing the enterokinase site did not help. When *D. nigrescens* β-glucosidase was tried

in the same plasmids, the same problems were seen. When the *D. cochinchinensis* β-glucosidase/dalcochinase enzyme that had been expressed in *P. pastoris* media was purified by standard chromatography methods (Charoenrat et al., 2006) was sequenced, it was found to start from a site 12 amino acid residues later than the protein in the plant, where the contruct was intended to start. Therefore, it seemed that the protein contained a cleavage site that was recognized by a protease in the *Pichia* cells or media. So, a new construct was made with the N-terminus of the cDNA removed so that the tag was connected to the cDNA sequence starting one residue after the cleavage site. This system allowed both the *D. cochinchinensis* and the *D. nigrescens* Dnbglu2 enzymes to be produced in active forms that could be purified by IMAC.

In fact, the purification of dalcochinase from *P. pastoris* media by expanded bed chromatography and gel filtration was efficient (Charoenrat et al., 2006), so the need for the use of the IMAC purification system may be unclear. However, we intended to mutate the *Dalbergia* β-glucosidases to test their activities, so it was necessary to have a purification system that would not be changed by changes in the protein. In fact, we were later able to make some mutations of the *D. cochinchinensis* β-glucosidase to make it more similar to the Dnbglu2 β-glucosidase and test their activities by using the N-terminal His-tag and IMAC purification system (Chuankhayan et al., In press). Initially, we intended to do this kind of work on this grant, but due to cuts in the budget, we had to find other funding to allow us to do this. However, the result is that the combination of this work and work on other grants allowed us to show that some residues in the active site of the *Dalbergia* β-glucosidases seem to contribute to their different specificities for different substrates, though other determinants of specificity remain to be determined.

The expression of the *D. nigrescens* β-glucosidase had its own problems, since the initially cloned cDNA could not produce active β-glucosidase despite our attempting yeast and bacterial systems, so we had to clone a new cDNA. In fact, the new cDNA, *Dnbglu2*, is clearly derived from another gene and has just over 80% amino acid sequence identity with the protein product of the previously cloned *Dnbglu1* cDNA. Though the protein seemed perhaps a bit different from the protein purified from the plant, based on the sequences of a few peptides derived from the protein purified from the plant, the activity was similar, in that it could efficiently hydrolyze isoflavonoid 7-O-β-D-glucosides and 7-O-β-D-acuminosides, which dalcochinase could not hydrolyze as well. So, it provided a convenient system to compare to the *D. cochinchinensis* dalcochinase to see what residues were likely to determine the substrate-specificity differences between these enzymes. Initially, only two residues that

had be identified as affecting aglycone specificity were found to be different between these two proteins, which are at amino acid residues 454 and 455 (Chuankhayan et al., 2007b). The dalcochinase has A454 and E455, while Dnbglu2 has S454 and G455. Thus, the system we developed could be used to test these residues by mutating the dalcochinase residues to Dnblgu2 residues at those positions. This showed that the double mutant could greatly increase the activity of the *D. cochinchinensis* dalcochinase toward the *D. nigrescens* isoflavonoid diglycoside substrates, though not to the same activity levels seen in Dnbglu2, suggesting other residues in the protein may also be involved. This result was similar to mutations of maize Glu1 to dalcochinase, where changing the corresponding two residues to those of sorghum dhurrinase allowed hydrolysis of dhurrin, at 3% the level of the dhurrinase itself (Verdoucq et al., 2004). Thus, the expression system produced in this work allowed useful structure-function analysis to be performed.

In the final section of this grant, the production of a Gateway expression system, was largely coordinated with a project on rice β-glycosidase gene function from the National Center for Genetic Engineering and Bioinformatics. So, one rice β-glucosidase gene, *Os4bglu12*, proved to be a covenient gene to test the system. This gene had the highest similarity to the protein sequence determined from a rice β-glucosidase purified from rice seedling cell walls (Akiyama et al., 1998). When this protein was put into the system, it could be transferred to several expression systems, though, of course, it took the research assistant much longer to test the systems than do the cloning. Nonetheless, the fact that it could be transferred to different systems in one step without analysis of the sequence for appropriate restriction sites saved a great deal of time. The maltose binding protein fusion from a *tac* promoter and thioredoxin fusions from the T7 and *araBAD* promoters were evaluated to show the T7 driven thioredoxin-tagged protein produced in Origami cells produced the highest activity for *E. coli* production. The starting clone was also transferred to the pPICZαBNH8/DEST vector and was successfully expressed in *P. pastoris*. After this, several other rice glycosyl hydrolase family 1 and family 35 β-glucosidases, β-mannosidases, and β-galactosidases could be successfully expressed in this system. In addition, we were able to express human α-galactosidase from the GalA gene in this system to allow a rapid analysis of a mutations effect on the protein function.

Despite the success of the Gateway system we set-up in producing proteins, roughly half of the GH family 1 enzymes that we tried to express in this system could not be expressed. The reason for this is unclear, since it did not seem to be obviously related to protein folding or uncommon codon usage. It might have been expected that codon usage of

unstability would have resulted in premature termination of the protein chain or cleavage of the target protein from the tag, therefore leaving the tag, but even the thioredoxin tag was not seen in the *E. coli* lysates. Strangely, when the proteins with this problem were cloned into the pPICZαBNH8/DEST pichia expression vector, they failed to express in *P. pastoris*, as well. In later work, Mariena Ketudat-Cairns' group tried to express one protein, Os11BGlu36, in several other bacterial and *Pichia* systems and even *in vitro* without success. However, a protein with a C-terminal green fluorescent protein (GFP) was produced in transient expression in plant cells, as indicated by the targeting of the GFP to the compartment expected for this protein. It is possible that these proteins are toxic to bacteria and yeast for some reason, but even tightly controlled expression using pLysS cells that make T7 lysozyme to suppress leaky T7 polymerase activity (Studier, 1991) did not allow expression of the proteins, so it is unlikely unless they are extremely toxic. Further investigation of these genes may be done in the future to see what prevents them from being expressed in the standard systems, thereby allowing the problem to be avoided in order to produce more proteins in recombinant systems.

On the whole, this project produced a large gain in our ability to express plant glycosyl hydrolases in recombinant systems. Though the sparse funding did not allow a concentrated project to produce an individual paper, the work contributed to the publication of at least 5 papers, in addition to helping with the training of two Ph.D. students. We now have several new vectors for expression and have also gained significant knowledge about the problems associated with expression of plant proteins in recombinant systems and some strategies for solving some of these problems.

4.2. Conclusions and Comments

In this project, we cloned and sequenced a new *Dalbergia nigrescens* Kurz β-glucosidase cDNA, *Dnbglu2*, and developed a way to recombinantly express this protein and the closely related *D. cochinchinensis* dalcochinase in active form with polyhistidine tags for purification. In addition, we developed a system for rapid screening of recombinant expression systems for proteins of interest. In doing so, we learned a large amount about recombinant expression of eukaryotic proteins in microbial systems.

The proteins produced ranged from isoflavonoid β-glucosidases from Thai legumous trees to rice cell wall β-glucosidase to human α-galactosidase. The *Dalbergia* β-glucosidases showed kinetic properties similar to those of enzymes purified from seeds (Toonkool et al., 2006; Chuankhayan et al., 2005, 2007b), while the rice Os4Bglu12 β-

glucosidase was found to have similar hydrolytic properties whether it was expressed in *E. coli* or *P. pastoris*. The expression of the human enzyme allowed a mutation to be evaluated to show it is likely to cause the Fabry disease in a Thai patient. In addition, the *D. nigrescens* β-glucosidase has been shown to release soy isoflavone phytoestrogens from their glycosides (Chuankhayan et al., 2007a), which may allow its application to increase soy food value. So, its recombinant production may provide a good source for its use in the food and feed industry. Thus, the infrastructure laid in this project could contribute to analysis of agricultural, industrial and medical problems.

The project was not well funded, so it had to be combined with other funds to produce publications, which resulted in its contributing to 5 international publications, in addition to 2 Ph.D. theses. Therefore, in terms of output, this project was quite successful, despite the funding limitations. It was also successful in terms of building infrastructure for further studies in the laboratory, which ultimately resulted in the publications. As the overall goal of the group project was to build expertise and knowledge in recombinant protein production, this is likely the main achievement of this project.

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from *Dalbergia cochinchinensis* Pierre, in yeast and bacterial hosts. *Prot. Express. Purif.* **48**, 195-204, doi: 10.1016/j.pep. 2006.05.011

Wattanasirichaigoon D., Svasti J., Cairns JRK, Tangnararatchakit K, Visudtibhan A, Keeratichamroen S, Ngiwsara L, Khowsathit P, Onkoksoong T, Lekskul A, Mongkolsiri D, Jariengprasert C, Thawil C, Ruencharoen S. 2006. Clinical and Molecular Analysis of an Extended Family with Fabry Disease. *J. Med. Assoc. Thailand.* **89**, 1528-1535.

Xu Z., Shih M.C., Poulton J.E. (2006) An extracellular exo-beta-(1,3)-glucanase from *Pichia pastoris*: purification, characterization, molecular cloning, and functional expression. *Prot. Express. Purif.* **47**, 118-27.

Yoshikawa M, Murakami T, Kishi A, Sakurama T, Matsuda H, Nomura M, Matsuda H, Kubo M 1998. Novel indole S,O-bisdesmoside, calanthoside, the precursor glycoside of tryptanthrin, indirubin, and isatin, with increasing skin blood flow promoting effects, from two *Calanthe* species (Orchidaceae). *Chem Pharm Bull (Tokyo)* **46**, 886-8

Curriculum Vitae

1. ชื่อ นาย.....เจมส์..... นามสกุล .....เกตุทัต-คาร์นส์.....

Name: James Last name: Ketudat-Cairns

2. รหัสประจำตัวนักวิจัยแห่งชาติ **38-30-0103**

3. ตำแหน่งปัจจุบัน .....รศ. .....

4. ประวัติการศึกษา

Educational background

ปีที่จบการศึกษา Year of graduation	ระดับปริญญา (ตรี โท เอก และประกาศนียบัตร) Degrees obtained (Bachelor's degree and above)	อักษรย่อปริญญาและชื่อเต็ม Abbreviation of degree title	สาขาวิชา Field of study	วิชาเอก Major	ชื่อสถาบันศึกษา Name of degree granting institution	ประเทศ Country
1986	Bachelor of Science	B. Sc.	Biology	Biology	University of Puget Sound	USA
1993	Philosophical Doctorate	Ph. D.	Biology	Biology	University of California	USA

5. สาขาวิชาที่มีความชำนาญพิเศษ ระบุสาขาวิชา

Fields of specialization

Protein Chemistry and Biochemistry, Cell and molecular biology, Bioinformatics

6. ประสบการณ์ที่เกี่ยวข้องกับงานวิจัยทั้งภายในและภายนอกประเทศ : ระบุสถานภาพในการทำวิจัยว่าเป็นหัวหน้าโครงการ หรือ ผู้ร่วมวิจัยในแต่ละเรื่อง

6.1 งานวิจัยที่เสร็จแล้ว : ชื่อเรื่อง ปีที่พิมพ์และสถานภาพในการทำวิจัย

List of completed research projects : title, year of publication and status of each project

1. Principle investigator: Isolation and Characterization of Rice β-glucosidase cDNAs Sept. 2000-August, 2002 Thailand Research Fund BGJ Grant (300,000 baht).
2. Principle Investigator: Isolation and characterization of Glycosyl Hydrolases from Thai Plants. Sept. 1996 – Aug., 1999. (Thai Research Fund Grant RSA 011/2539)
3. Coinvestigator: Cloning and sequence anaylsis of the β-glucosidase/β-fucosidase enzyme from Thai Rosewood. Paper accepted for publication, J. Biochem. Dec. 2000.
4. Coinvestigator: Modeling of the disulfide knot in protein structure. Project not published.
5. Coinvestigator (student & postdoc): Vitamin K-Dependent γ-carboxylation of Bone Gla Protein. Publication 1: 1989. Thesis: 1993, Publication 2: 1994.

6.2 งานวิจัยที่กำลังดำเนินการ : ชื่อเรื่องและสถานภาพในการทำวิจัย

1. Principle investigator: Investigation of Rice β-glycosidase gene functions. July, 2003-June, 2006 NSTDA BIOTEC Grant (3,220,000 baht).
2. Principle Investigator: Protein structure-function relationships in plant β-glucosidases BRG4780020 August, 2004-August, 2007, Thailand Research Fund (2,000,000 baht)

3. Coinvestigator: Analysis of Proteins, Blood, and Body Fluids for Diagnosis, Counseling and Treatment of Genetic Diseases 1997-2000. (Chulabhorn Research Institute grant)
4. Coinvestigator: Screening, Purification and Characterization of Glycosides from Thai Plants for Medicinal Purposes. 1997-2000. (NRC grant & Chulabhorn Research Institute 97-98).
5. Coinvestigator: Abnormalities in proteins and enzymes in relation to cancers found in Thailand. (Chulabhorn Research Institute grant)

Publications (Journal Articles)

1. Chuankhayan P, Rimlumduan T, Tantanuch W, Kongsaeree PT, Metheenukul P, Svasti J, Jensen ON, Ketudat Cairns JR (2007) Functional and structural differences between isoflavonoid β-glycosidases from *Dalbergia* sp. *Archives of Biochemsitry and Biophysics* 468 (2), 205-216. doi:10.1016/j.abb.2007.09.015

2. Opassiri R, Pomthong B, Akiyama T, Nakphaichit M, Onkoksoong T, Ketudat-Cairns M, and James R Ketudat Cairns (2007) A stress-induced rice β-glucosidase represents a new subfamily of glycosyl hydrolase family 5 containing a fascin-like domain. *Biochemical Journal* **408** (2), 241-249. doi:10.1042/BJ20070734

3. Suwannarat P, Keeratichamroen S, Wattanasirichaigoon D, Ngiwsara L, Ketudat Cairns J R, Svasti J, Visudtibhan A, Pangkanon S. (2007) Molecular characterization of type 3 (neuronopathic) Gaucher disease in Thai patients. *Blood Cells, Molecules and Diseases* **38,** 348-352. doi:10.1016/j.bcmd.2007.06.015 (ISI IF 2.427, 2005)

4. Chantarangsee M, Payakapong W, Fujimura T, Fry SC, Ketudat Cairns JR (2007) Molecular cloning and characterization of β-galactosidases from germinating rice *(Oryza sativa) Plant Science* **173**, 118-134. doi:10.1016/j.plantsci.2007.04.009

5. Hommalai G, Stephen G. Withers SG, Chuenchor W, Ketudat Cairns JR, Svasti J. (2007). Enzymatic synthesis of cello-oligosaccharides by mutated rice β-glucosidases. *Glycobiology* **17**, 744-753. doi: 10.1093/glycob/cwm039 (ISI IF 3.512, 2005)

6. Keeratichamroen S, Ketudat Cairns JR, Sawangareetrakul P, Liammongkolkul S, Champattanachai V, Srisomsap C, Kamolsilp M, Wasant P, Svasti J. (2007) Novel mutations found in two genes of Thai patients with isolated methylmalonic acidemia. *Biochemical Genetics* **45**, 421-430. doi: 10.1007/s10528-007-9085-y

7. Chuankhayan P, Rimlumduan T, Svasti J, Ketudat Cairns JR. 2007. Hydrolysis of Soybean Isoflavonoid Glycosides by *Dalbergia* β-Glucosidases. *Journal of Agricultural and Food Chemistry* **55**, 2407-2412.

8. Opassiri R, Pomthong B, Okoksoong T, Akiyama T, Esen A, Ketudat Cairns JR. 2006. Analysis of Rice Glycosyl Hydrolase Family 1 and Expression of *Os4bglu12* β-Glucosidase *BMC Plant Biology* **6**, 33.

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14. Opassiri R, Hua Y, Wara-Aswapati O, Akiyama T, Svasti J, Esen A, and Ketudat Cairns JR. 2004. β-Glucosidase, exo-β-glucanase and pyridoxine transglucosylase activities of rice BGlu1. *Biochemical Journal* **379**, 125-131. DOI: 10.1042/BJ20031485.

15. Opassiri R, Ketudat Cairns JR, Akiyama T, Wara-Aswapati O, Svasti J, and Esen A. 2003. Characterization of a rice β-glucosidase genes highly expressed in flower and germinating shoot *Plant Science* **165**, 627-638.

16. Kaomek M, Mizuno K, Fujimua T, Sriyotha P, Ketudat Cairns JR. 2003. Cloning, Expression and Characterization of an Anti-Fungal Chitinase from *Leucaena leucocephala* de Wit. *Bioscience, Biotechnology and Biochemistry* **67**, 667-676.

17. Champattanachai V, Ketudat Cairns, JR, Shotelersuk V, Kerratichamreon S, Sawangareetrakul P, Srisomsap C, Kaewpaluek V, Svasti J. 2003. Novel Mutations in a Thai patient with methylmalonic acidemia. *Molecular Genetics and Metabolism* **79**, 300-302.

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19. Ketudat Cairns JR, Champattanchai V, Srisosap C, Thiede B, Wittman-Liebold B, Svasti J. 2000. Sequence and Expression of Thai Rosewood β-Glucosidase/β-Fucosidase, a Family 1 Glycosyl Hydrolase Glycoprotein. *Journal of Biochemistry* **128**, 999-1008.

20. Ketudat Cairns JR, Chantarangsee M, Chaiwangrad S, Phawong J. 1999. Primary Structure-Based Screening for Glycosyl Hydrolases in Thai Plants. *Thai Journal of Biotechnology* **1**, 20-30.

21. Svasti J, Srisomsap C, Surarit R, Techasakul S, and Ketudat-Cairns JR. 1998. Characterization of a Novel Rotenoid-β-Glucosidase and its Natural Substrate from Thai Rosewood. *Journal of Pure and Applied Chemistry* 70 (11), http://www.iupac.org/symposia/proceedings/phuket97/svasti.html.

22. Cairns JR, Price PA. 1994. Direct Demonstration That the Vitamin K-Dependent Bone Gla Protein Is Incompletely γ-Carboxylated in Humans. *Journal of Bone and Mineral Research* **9**, 1989-1997.

23. Cairns JR, Williamson MK, Price PA. 1991. Direct Identification of γ-Carboxyglutamic Acid in the Sequencing of Vitamin K-Dependent Proteins. *Analytical Biochemistry* **199**, 93-97.

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Education:

1988	B.Sc.	Biology (Plant Science and Technology) Minor in Chemistry Chiang Mai University, Thailand G.P.A. 3.24
1995	Ph.D.	Biology (Plant Molecular Biology and Genetic Engineering) University of California San Diego, USA G.P.A. 4.00

Awards, Scholarships & Training courses:

1988	Prof. Dr. Dhab Nelanithi Foundation Award
1988-1990	Scholarship from The Institute for Promotion of Teaching Science and Technology (IPST), Thailand
1990-1991	Scholarship from Biology Department UCSD, USA
1991-1992	Graduate Student Fellowship UCSD, USA
1992-1995	Genetics Training Grant from National Institute of Health (NIH), USA
1995	28/8-13/10 International Training Program (ITP) in Biotechnology at Gesellschaft fur Biotechnologische Forschung (GBF), Braunschweig, Germany
2001	10-21/9 ICRO-UNESCO International Training course on RNA and Biotechnology at Chinese Academic of Science, Shanghai, China

Experience:

1988-1995	Research Assistance, UCSD
1989-1994	Teaching Assistance, UCSD
1990-1995	Teaching Assistance Trainer, Department of Biology, UCSD
1995	4/11-16/12 Head of the Business Center for WorldTech'95, Thailand
1995-1998	Instructor, Suranaree University of Technology
1997	Secretariat of the JSPS/NRCT Biotechnology Conference, Thailand
1998-2000	Secretariat of the SUT Biotechnology Graduate Curriculum Development
1998-present	Assistant Professor, Suranaree University of Technology

Membership: Thai Society for Biotechnology (Society committee 1998-2002, 2 terms)
Thai Society for Genetics

Research Grants Awarded:

1995-1997	200,000 Baht from National Science and Technology Development Agency (NSTDA) for The Production of Taq DNA polymerase for Research and Laboratory Classes.
1997-1999	1,418,080 Baht from NSTDA for Tilapia Sex Chromosome Identification Using DNA Marker.
1999-2001	400,000 Baht from Suranaree University of Technology The study of Thai *Dendrocalamus asper* (bamboo) genetic maps
2000-2002	450,000 Baht from Suranaree University of Technology Expression of B-glucoside from Thai Rosewood in *Pichia pastoris*
1997-1999	3,500,000 Baht from Ministry of University Affairs, Thailand Biotechnology Graduate Curriculum Development
2000-2005	10,000,000 Baht from Ministry of University Affairs, Thailand Development of Sustainable International Biotechnology Graduate Education

Publications & Paper presented:

Opassiri, R., Pomthong, B., Akiyama, T., Nakphaichit., M., Onkoksoong, T, **Ketudat-Cairns, M.**, and Ketudat Cairns, J.R. (2007) A stress-induced rice β-glucosidase represents a new subfamily of glycosyl hydrolase family 5 containing a fascin-like domain. *Biochemical Journal* **408** (2), 241-249. doi:10.1042/BJ20070734

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Toonkool, P., Metheenukul, P., Sujiwattanarat, P., Paiboon, P., Tongtubtim, N., **Ketudat-Cairns, M.**, Ketudat-Cairns, J., Svasti, J. (2006) Expression and purification of dalcochinase, a β-glucosidase from *Dalbergia cochinchinensis* Pierre, in yeast and bacterial hosts. *Protein Expression and Purification* **48**, 195-204, doi: 10.1016/j.pep. 2006.05.011

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Laowtammathron, C., Lorthongpanich, C., **Ketudat-Cairns, M.**, Hochi, S., Parnpai, R. 2005. Factors affecting cryosurvival of nuclear-transferred bovine and swamp buffalo blastocysts: the effects of hatching stage, linoleic acid-albumin in culture medium, and Ficoll supplementation to vitrification solution. *Theriogenology* 64, 1185-1196

Charoenrat, T., Vanichsrirattana, V., and **Ketudat-Cairns** (2004) Recombinant β-glucosidase Production by *Pichia pastoris*: Influence of pH. *Thai Journal of Biotechnology* 5 (1) 51-55

Lorthongpanich, C., Laowtammathron, C., Muenthaisong, S., Vetchayan, T., **Ketudat-Cairns, M.**, Likitdecharote, B. and Parnpai, R. (2004). *In vitro* development of enucleated domestic cat oocytes reconstructed with skin fibroblasts of domestic and leopard cats. *Reprod. Fert. Dev.* 16: 149.

Kanchanatawee, S., Wanapu, C. and **Ketudat-Cairns, M.** (2000) Biotechnology Graduate Education in Thailand. *Thai Journal of Biotechnology* 2 (1): 55-62

Carlini, L.E., **M. Ketudat**, R.L. Parsons, S. Prabhakar, R. J. Schmidt and M. J. Guiltinan (1999) The maize bZIP protein orthologue of EmBP-1: Activation of gene expression in yeast from an O2 box and localization of a bipartite nuclear localization signal (NLS). *Plant Molec. Biol.*41: 339-349. (M. Ketudat and L. Carlini are Co-first authors)

Ketudat-Cairns, M. (1998) Biotechnology and Daily Life. *Suranaree J. Sci Technol* 5:208-211

Manakasem Y., Sornsuk P., and **Ketudat-Cairns M.** (1998) A survey of the Status and Problems of the Vegetable and Fruit Production and Post-Harvest Handling System in Nakhon Ratchasima Province. *Suranaree J. Sci Technol* 5:95-100

Schmidt, R. J., Pysh, L. D., **Ketudat, M.**, Parsons, R. L., and Hoschek, G. (1994) bZIP Proteins Regulating Gene Expression in Maize Endosperm. In *Molecular Genetic Analysis of Plant Metabolism and Development* (G. Coruzzi and P. Puigdomenech, eds.) NATO ASI Proceedings

Schmidt, R. J., **Ketudat, M.**, Aukerman, M. J., and Hoschek, G. (1992) Opaque-2 is a Transcriptional Activator that Recognizes a Specific Target Site in 22-kD Zein Genes. *Plant Cell* 4:701-709

Curriculum vitae

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4. Educational Background

Year of Completion	Degree	Abbreviation of Degree	Field	Major	Name of Granting Institution	Country
1998	Bachelor of Science	B. Sc.	Biology	Biology	Khon Kaen Univ.	Thailand
2003	Doctor of Philosophy	Ph. D.	Environmental Biology	Environmental Biology	Suranaree Univ. of Technology	Thailand

5. Areas of specialization

Plant molecular biology and biochemistry, structure and function of enzymes, Functional genomics

6. Research Experience

7.1 Completed Research Projects.

- Coinvestigator: Beta-glucosidase from rice; Sept., 2000 to February, 2003. Funding: the Thailand Research Fund BGJ Grant; Results: publication of one paper in *Biochemical Journal,* 2004

- Coinvestigator: Isolation and characterization of rice beta-glucosidase; June, 1999 to August, 2003 Royal Golden Jubilee Scholarship Project from the Thailand Research Fund. Output: 1 paper in *Plant Science*, 2003

- Coinvestigator: Investigation of rice beta-glycosidase gene functions; June, 2003-May, 2006. Funding: National Center for Genetic Engineering and Biotechnology, National Science and Technology Development Agency.

-Coinvestigator: Protein structure-function relationships in plant β-glucosidases BRG4780020, August, 2004-August, 2007, Thailand Research Fund

7.2 Current Research Projects:

- Primary Investigator: Rice blast response. 2005-2008; SUT/NRCT grant.

- Co-principle investigator: Structural studies of carbohydrate active enzymes from rice. Nov., 2006-Nov., 2008. National Synchrotron Research Center.

-Coinvestigator: Protein structure-function relationships in plant β-glucosidases II, BRG508007, July, 2007-July, 2010. Thailand Research Fund

8. Publications:

1. Opassiri R, Pomthong B, Akiyama T, Nakphaichit M, Onkoksoong T, Ketudat-Cairns M, and James R Ketudat Cairns (2007) A stress-induced rice β-glucosidase represents a new subfamily of glycosyl hydrolase family 5 containing a fascin-like domain. *Biochemical Journal* **408** (2), 241-249. doi:10.1042/BJ20070734

2. Suginta, W., Songsiriritthigul, C., Kobdaj, A., Opassiri, R., and Svasti, J. 2007. Mutations of Trp275 and Trp397 altered the binding selectivity of Vibrio carchariae chitinase A. *Biochim. Biophys. Acta* **1770** (8), 1151-1160.

3. Opassiri R, Pomthong B, Okoksoong T, Akiyama T, Esen A, Ketudat Cairns JR. 2006. Analysis of Rice Glycosyl Hydrolase Family 1 and Expression of *Os4bglu12* β-Glucosidase *BMC Plant Biology* **6**, 33.

4. Chuenchor W, Pengthaisong S, Yuvaniyama J, Opassiri R, Svasti J and Ketudat Cairns JR. 2006. Purification, Crystallization and Preliminary X-ray Analysis of Rice BGlu1 β-Glucosidase with and without 2-deoxy-2-fluoro-β-D-glucoside Inhibitor *Acta Crystallographica Section F* **62**, 798-801. doi:10.1107/S1744309106027084.

5. Opassiri R, Hua Y, Wara-Aswapati O, Akiyama T, Svasti J, Esen A, and Ketudat Cairns JR. 2004. β-Glucosidase, exo-β-glucanase and pyridoxine transglucosylase activities of rice BGlu1. *Biochemical Journal* **379**, 125-131. DOI: 10.1042/BJ20031485.

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