รหัสโครงการ SUT1-102-48-36-17

รายงานการวิจัย

การตรวจหาด้วยเอนไซม์และการศึกษาสารประเภทไกลโคไซด์จากพืชไทย

Enzymatic screening and characterization of Thai plant glycosides

ได้รับทุนอุดหนุนการวิจัยจาก
มหาวิทยาลัยเทคโนโลยีสุรนารี

ผลงานวิจัยเป็นความรับผิดชอบของหัวหน้าโครงการวิจัยแต่เพียงผู้เดียว

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ผลงานวิจัยเป็นความรับผิดชอบของหัวหน้าโครงการวิจัยแต่เพียงผู้เดียว

9/2552

Acknowledgement กิตติกรรมประกาศ

The researchers on this project are grateful to the students who worked on this research and the others who advised and supported the research. Phimonphan Chuankhayan isolated and determined the structures of the *Dalbergia nigrescens* isoflavone glycosides with the help of Yanling Hua. Dr. Chuankhayan also characterized the hydrolysis of soy isoflavones in extracts and soy flour suspensions, and helped with initial screening for glycosides in rice extracts. Thipwarin Rimlumduan helped with expression of *Dalbergia* β-glucosidases in *Pichia pastoris*, while Janjira Maneesan and Sompong Sansenya helped with screening of extracts of various rice tissues for glycosides and Ms. Maneesan also purified rice glycosides, with some assistance from Dr. Takashi Akiyama of the National Agricultural Research Center for the Hokkaido region and Dr. H. Jin of Sapporo, Japan. Por-gnam Prasert also helped with purification of *Dalbergia* glycosides. Dr. Rodjana Opassiri oversaw and provided support for the Os4BGlu12 screening of rice extracts and intial purification efforts for the glycosides from the shoot and root extract. Dr. Pichaya Trakanrungroj is thanked for helpful discussion and helping to oversee one extraction of rice glycosides, while Dr. Santi Sakdarat is thanked for confirming the *Dalbergia* glycoside structures. Advice was provided along the way by Prof. Dr. M.R. Jisnuson Svasti, Dr. Chantragan Srisomsap, Dr. Palangpol Kongsaeree and Dr. Prachumporn Kongsaeree.

This research project received a research grant from Suranaree University of Technology, beginning in the fiscal years 2005, 2006 and 2007. (การวิจัยครั้งนี้ได้รับทุนอุดหนุนการวิจัยจากมหาวิทยาลัยเทคโนโลยีสุรนารี ปีงบประมาณ2549-2551).

บทคัดย่อ (Thai abstract)

โครงการนี้มีจุดประสงค์ในการใช้เอนไซม์บีต้ากลูโคซิเดสที่ผลิตได้ในห้องปฏิบัติการเพื่อบ่งชี้และหาคุณสมบัติของไกลโคไซด์ในสารสกัดจากพืช รวมถึงศึกษากลไกการทำงานของเอนไซม์เหล่านี้ ในการวิจัยได้แบ่งการทดลองออกเป็นสองส่วน ส่วนที่หนึ่งได้ทำการศึกษาคุณสมบัติของสารสกัดจากต้นฉนวน (*Dalbergia nigrescens*) และใช้สารสกัดที่ได้มาทดสอบกลไกการทำงานของบีต้ากลูโคซิเดสต่อไอโซฟลาโวน ส่วนที่สองได้บ่งชี้ถึงไกลโคไซด์จากข้าวที่น่าจะเป็นสารตั้งต้นของเอนไซม์บีต้ากลูโคซิเดสที่ศึกษาในห้องปฏิบัติการชีวเคมี มทส

ในส่วนแรก ได้ทำการยืนยันโครงสร้างของไอโซฟลาโวนอยด์ไกลโคไซด์ของดาลไนกระเซน และดานไนกริน หรือ ดาลไนกรีดิน และพบว่าเอนไซม์นี้สามารถแตกพันธะระหว่างกลูโคสและไอโซเฟลโวน 7-O เพื่อปลดปล่อยน้ำตาลโมเลกุลคู่อะดูมิโนส นอกจากนี้ยังพบว่าริคอมบิแนนท์เอนไซม์ Dnbglu2 ก็สามารถทำได้เช่นเดียวกัน ถึงแม้การแสดงความสามารถในการแตกพันธะของสารตั้งต้นทั้งสองนี้เกิดขึ้นได้ดี แต่พบว่าความสามารถในการแตกพันธะของไอโซเฟลโวน 7-O กลูโคไซด์ไดอะซินและเจนิสตีนจากถั่วเหลืองสูงกว่าเมื่อดูจากค่า k_{cat}/K_m เอนไซม์บีต้ากลูโคซิเดสจากต้นฉนวนสามารถแตกพันธะไอโซเฟลโวนในแป้งถั่วเหลืองและสารละลายแป้งได้ จึงสามารถใช้เอนไซม์นี้ในการเพิ่มไอโซเฟลโวนอิสระในผลิตภัณฑ์ถั่วเหลืองได้ เอนไซม์นี้สามารถแตกพันธะมาโลนิลและอเซติลเจนิสตีน และไดอาซีนได้เช่นเดียวกับที่สามารถเป็นตัวรับหมู่อพิโอซิลในตำแหน่ง 6'-O ของกลูโคสจากกลุ่มไกลโคไซด์ที่มีอยู่ในธรรมชาติ ผลจากการค้นพบนี้ได้รับการตอบรับให้ตีพิมพ์ในวารสารระดับนานาชาติสามบทความ

ในส่วนที่สอง Os4BGlu7 และ Os4BGlu12 จากข้าว ถูกใช้ในการค้นหาสารตั้งต้นในธรรมชาติของข้าวโดยการสกัดสารจากข้าวที่อายุต่าง ๆ คือ ต้นอ่อน รากอ่อน ต้นข้าวที่กำลังออกรวง โดยแยกสารออกมาจากส่วนที่เป็นต้นและรากด้วยเมธานอล ในเบื้องต้นพบว่าเอนไซม์ทั้งสองสามารถแตกพันธะไกลโคไซด์ที่เรืองแสงในส่วนของต้นอ่อน อย่างไรก็ตามยังไม่สามารถที่จะสกัดสารนี้ได้เพียงพอสำหรับการหาโครงสร้างของสารนี้ จากนั้นจึงทำการค้นหาสารตั้งต้นที่จำเพาะต่อเอนไซม์ Os4BGlu7 และพบว่าสามารถแตกพันธะของไกลโคไซด์จากรากอ่อน ใบแก่ และต้นได้ ไกลโคไซด์เหล่านี้ถูกทำให้บริสุทธิ์ผ่านโครมาโตกราฟีโดยใช้คอลัมน์ LH20, TLC และ reverse phase HPLC ซึ่งได้ไกลโคไซด์บริสุทธิ์จำนวนหนึ่งและไม่บริสุทธิ์อีกจำนวนหนึ่ง ไกลโคไซด์ที่บริสุทธิ์สามชนิด พบว่ามีน้ำหนักโมเลกุลเท่ากับ 492 688 และ 688 จากการวิเคราะห์ผ่าน electrospray mass spectrometry จึงได้ตั้งชื่อว่า Cs/1D1 Cs/1D2 และ Cs/1D3 ตามลำดับ น้ำหนักโมเลกุลของ Cs/1D1 ใกล้เคียงกับ trihydrozyl dimethoxy glucoside เช่น tricin 4'-O-β-D-glucoside และ tricin 7-O- β-D-glucoside ที่เคยมีรายงานว่าพบในข้าว ในการทดลองเพื่อศึกษาโครงสร้างต่อโดยใช้ NMR จำเป็นต้องมีการเตรียมสารเหล่านี้ให้ได้ในปริมาณมาก การทดลองนี้พบว่าเอนไซม์บีต้ากลูโคซิเดสสามชนิดจากข้าวที่ทราบโครงสร้างแล้วคือ Os3BGlu6 Os3BGlu7 และ Os4BGlu12 สามารถแตกพันธะของ Cs/1D1 ในอัตราที่ใกล้เคียงกัน แต่การแตกพันธะของ Cs/1D2 ถูกทำลายอย่างรวดเร็วโดยเอนไซม์ Os3BGlu6 และ Cs/1D3 ถูกแตกพันธะได้เร็วโดยเอนไซม์ Os4BGlu12 ดังนั้นจึงสามารถสรุปได้ว่า แม้ไกลโคไซด์เหล่านี้จะมีขนาดโมเลกุลเท่ากัน แต่เอนไซม์ Os3BGlu6 และ Os4BGlu12 สามารถแยกความแตกต่างของไกลโคไซด์ทั้งสองชนิดได้

การศึกษาในอนาคตควรเตรียมสารไกลโคไซด์ Cs/1D1 Cs/1D2 และ Cs/1D3 ในปริมาณมาก เพื่อใช้ในการศึกษาโครงสร้างและการแตกพันธะของสารทั้งสามชนิดนี้โดยเอนไซม์บีต้ากลูโคซิเดสจากข้าว ซึ่งจะสามารถทำให้ทราบหน้าที่ของเอนไซม์และไกลโคไซด์ในข้าวได้

Abstract:

This project was aimed at the use of β-glucosidase enzymes produced in our lab to identify and characterize glycosides in plant extracts and the mechanism of enzyme hydrolysis of these glycosides. It was broken down into two parts, 1) completion of the characterization of *Dalbergia nigrescens* natural substrates and the use of *D. nigrescens* β-glucosidase to hydrolyze natural isoflavone glycosides, and 2) the identification of rice glycosides that may serve as natural substrates for the rice enzymes characterized in our laboratory.

In the first part, we were able to confirm the structures of two isoflavonoid glycosides, dalnigrecin [dalpatein 7-O-(1,6)-β-D-apiofuranosyl-β-D-glucopyranoside], and dalnigrin [7-O-β-D-apiosyl-(1,6)-β-D-glucosyl-7-hydroxy-2',4',5',6-tetramethoxyisoflavone] or dalnigrein 7-O-(1,6)-β-D-apiofuranosyl-β-D-glucopyranoside]. It was also shown that the enzyme hydrolyzed the bond between the glucose and the isoflavone 7-O to release the disaccharide acuminose. This was also found to be true for the recombinant *D. nigrescens* β-glucosidase Dnbglu2, which showed high activity toward these two natural substrates, although it hydrolyzed the soy isoflavone 7-O-glucosides diadzin and genistin with higher efficiency (k_{cat}/K_m values). The *D. nigrescens* β-glucosidase could also hydrolyze soy isoflavones in soy flour extracts and suspensions, suggesting it could be used to increase the levels of free isoflavones in soy products. The enzyme could hydrolyze malonyl and acetyl genistin and diadzin, consistent with its acceptance of an apiosyl group at the 6'-O position on the glucosyl moiety of the natural product glycosides. This work, in addition to suggesting the use of D. nigrescens for soy food processing, generated 3 international journal papers.

In the second part, the rice β-glucosidases Os3BGlu7 and Os4BGlu12 were used to screen for natural glycoside substrates in methanolic extracts of rice seedling shoot and root and mature rice plant at flowering stage shoot and root fractions. Initial experiments indicated that both compounds could hydrolyze a fluorescent glycoside in seedling shoots, but insufficient quantity and purity of glycoside was generated to determine the structure. Since the Os4BGlu12 enzyme is more stable for storage, it was used for further screening and was found to hydrolyze glycosides in seedling roots and mature leaf and stem. The leaf and stem glycoside fraction was purified by LH20 column chromatography, preparative TLC and reverse phase HPLC to give 3 apparently pure glycosides from one TLC spot from a pool of LH20 fractions, as well as some as yet impure glycosides. The three purified components, Cs/lD1, Cs/lD2, and Cs/lD3 were analyzed by electrospray mass spectrometry to show they have apparent molecular masses of 492, 688 and 688, respectively. The mass of the Cs/lD1 matches that of a trihydroxyl dimethoxy glucoside, such as tricin 4'-O-β-D-glucoside and tricin 7-O-β-D-glucoside, which have been reported to occur in rice. Further preparations of large amounts of material will be needed for complete structure determination by NMR. It was found that three rice β-glucosidases with known structures, Os3BGlu6, Os3BGlu7 and Os4BGlu12, hydrolyzed Cs/lD1 at similar rates, but Cs/lD2 was hydrolyzed most rapidly by Os3BGlu6, while Cs/lD3 was hydrolyzed most rapidly by Os4BGlu12. Thus, despite the fact that they have the same mass, the Os3BGlu6 and Os4BGlu12 enzymes seem to differentiate these two compounds. Further studies of large scale preparations of Cs/lD1, Cs/lD2 and Cs/lD3 will allow structural characterization and kinetic studies of their hydrolysis by the rice β-glucosidases, which can tell about the functions of these enzymes and the glycosides.

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List of Abbreviations and Symbols

A	Absorbance
Amp	Ampicillin
ATP	Adenosine triphosphate
BSA	Bovine Serum Albumin
°C	Degrees celsius
cDNA	Complementary deoxynucleic acid
CTP	Cytosine triphosphate
DEPC	Diethyl pyrocarbonate
DNA	Deoxyribonucleic acid
DnS	*Dalbergia nigrescens* substrate
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
NMR	Nuclear Magnetic Resonance
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 those of vitamins, phytohormones, pigments, lignin precursors, volatiles involved in aroma production, pesticides and antifungal agents and a broad range of other compounds (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 (Rubinelli, Hu, Ma, 1998; Smith, Starrett, and Gross, 1998, Mizutani et al., 2002). Although many glycosidases 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), it is mainly the β-D-glucosidases and related enzymes that act on glycosides (Svasti et al., 1999; Arthan et al., 2002, 2006, Chuankhayan et al., 2005, 2007a, 2007b).

Many of these glycosides and their enzymes function in defense against herbivores and parasites in plants (Esen, 1993; Falk and Rask L. 1995; Morant et al., 2008). 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 may similarly release HCN in other plants (Poulton, 1990; Cicek and Esen, 1998; Morant et al., 2008). Other compounds, such as rotenone and rotenoids from *Dalbergia* species may also be toxic to insects (Abe *et al.*, 1985). Thus, these compounds are of interest in the control of insects.

Glycosides and the glycosidases that hydrolyze them have many functions and applications of current or potential economic significance to Thailand. For instance, since vitamins and beneficial phytoestrogens 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). In addition, the aroma of tea increases over time upon processing due to the gradual mixing of glycosides of aromatic compounds (especially primerverosides) with the enzyme(s) that hydrolyze them and release the aromatics (Mizutani *et al.*, 2002). Volatile compounds from flowers are also released from glycosides by β-glucosidases (Reuvni *et al.*, 1999).

Many glycosides, some with useful bioactivies have been characterized from *Dalbergia* sp. and related legumous trees found in Thailand. These include rotenone and rotenoid glycosides like amorphigenin-8'-β-D-glucoside and dalbin from *Dalbergaia monetaria* L (Abe *et al.*, 1985) and *Dalbergia latifolia* (Chibber and Khera, 1979) and dalcochinin-8'-O-β-D-glucoside from *Dalbergia cochinchinensis* Pierre (Svasti *et al.*, 1999), and other isoflavonoids like isocaviunin 7-gentiobioside from *Dalbergia sisso* (Sharma *et al.*, 1980) and 5-hydroxy-5,7-dimethoxy-4'-O-(6-O-β-D-furanosyl-β-D-glucopyranosyl) isoflavone from *Dalbergia nigra* (Mathias *et al.*, 1998). Additionally, many other compounds have been described from *Dalbergia* species that may potentially be O-glycosylated as well (Donnelly *et al*, 1965, 1968; Muangnoicharoen and Frahm, 1981; Rao and Rao, 1991; Bekker *et al*, 2001; Shirota *et al*, 2003). Since plant alkaloid synthesis and other metabolic pathways involve glycosylation to block certain reactions, followed by deglycosylation at the appropriate step (Warzecha *et al.*, 2000), it is anticipated many other glycosides remain to be found. Many of these glycosides and potential aglycones have been

shown to have biological activities such as being insecticidal to mosquito larvae, antioxidants, inhibitors of enzymes involved in disease, etc. Svasti *et al.* (1999) showed the enzyme which hydrolyzed a glycoside from a plant is a useful tool in identifying that glycoside and purifying it. They have since used the same β-glycosidase to identify new glycosides from other plants, such as torvasides A and H from *Solanum torvum* (Arthan *et al.*, 2002). Since the glycoside and aglycone often have quite different bioactivities, it is useful to have a gentle way to hydrolyze the glycoside to test both (Svasti *et al.*, 1999).

In addition to the identification of new glycosides, β-glucosidases can be used to convert known glycosides to useful forms, with one of the prominent targets being soy isoflavones. The soy isoflavones, predominantly diadzein, genestein and glycitein, have been shown to reduce the incidence of breast, prostrate and colon cancer (Barnes and Messina, 1991; Messina et al., 1994; Hendrich et al., 1994). Since they are phytoestrogens, soy isoflavones have also been considered as a potential treatment for menopausal women, since the normal estrogen-replacement therapy carries the risk of stimulating some forms of breast cancer (Song et al., 1999; Albertazzi and Purdie, 2002; Barnes, 2003). In addition, these isoflavones are antioxidants and have been reported to have anti-atherosclerotic properties and lower blood glucose and low-density lipoproteins, while increasing high-density lipoproteins, so they provide protection from coronary disease (Kwon et al., 1998; Chung et al., 2000; Demonty et al., 2003). Although there is some debate, due to the presence of bacterial and human β-glucosidases in the intestines, deglycosylating the glycosides of these isoflavones, daidzin, genestin, and glycitin, appears to improve their bioavailability (Barnes and Messina, 1991; Anderson et al., 1995; Bahram et al., 1996). Thus, β-glucosidases from almond and bacteria have previously been analyzed for their actions in releasing the free soy isoflavones from their glycosides, and showed varying degrees of efficiency (Hessler et al., 1997; Pandjaitan et al., 2000; Tsangalis et al., 2002; Ismail and Hayes, 2005).

Using the evolutionary relationship between these enzymes, we have cloned cDNA for β-galactosidases, β-glucosidases, and chitinases from various plants (Ketudat Cairns *et al.*, 2000, Ketudat-Cairns *et al*, 1999, Kaomek *et al.*, 2003, Opassiri *et al*, 2003, 2006, Chuankhayan et al., 2007b). The novel rotenoid β-glucosidase from *Dalbergia cochinchinensis* Pierre, dalcochinase, which was the first enzyme cloned in an effort with the Chulabhorn Research Institute Biochemistry laboratory, had a potentially useful substrate (Svasti *et al.*, 1998; Ketudat Cairns *et al.*, 2000). This dalcochinase can also be used for synthesis of β-glucosides of various alcohols (Lirdprapamongkol and Svasti, 2000; Svasti *et al*, 2003). A closely related β-glucosidase from *Dalbergia nigrescens* has also been cloned recently (Chuankhayan et al., 2007b). This enzyme was 81% 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. At the start of this project, we had still not completely defined the substrates for *Dalbergia nigrescens*, so this was one of the first activities of this project. We have previously expressed recombinant *D. cochinchinensis* enzyme in *P. pastoris*, however the low yields made it difficult to completely characterize and engineer the enzyme (Ketudat Cairns et al., 2000). Therefore, a new system was developed for the expression of this enzyme that allowed purification via an N-terminal His8-tag (Toonkool et al., 2006). This system ws also used to express *D. nigrescens* Dnbglu2 (Chuankhayan et al., 2007b), while several rice β-glucosidases and β-galactosidases and *Leucaena leucocephala* chitinase were expressed as N-terminal thioredoxin and His6-tagged fusion proteins in *Escherichia coli* in our group (Kaomek et al., 2003; Opassiri et al., 2003, 2006, 2007; Chantarangsee et al., 2007; Tantanuch et al., 2008; Seshadri et al., 2009). These glycosidases provided useful tools that could be used to screen for, characterize and hydrolyze natural glycosides in this work. Once the glycosides were found, they were purified for structure elucidation and used to characterize the substrate-specificity of various glycosidases.

1.2. Research Objectives (วัตถุประสงค์ของการวิจัย)

1.2.1. Production and purification of β-glucosidases from *Dalbergia* sp and rice we have previously cloned by recombinant methods or extraction from seeds.

1.2.2. Identification of natural glycoside substrates for the enzymes from their own plant source and from extracts of other plants, especially *Dalbergia nigrescens* and rice.

1.2.3. Purification of the glycosides by chromatography using enzymatic hydrolysis as an assay.

1.2.4. Determination of the structures of the glycosides.

1.2.5. Application of *Dalbergia* β-glucosidase to conversion of isoflavone glycosides in soy.

1.3. Scope of Research (ขอบเขตของการวิจัย)

Due to the limitation on funding, the project concentrated on glycoside-related aspects of other projects. These included finalizing the structure of isoflavonoid glycosides from *D. nigrescens* and their purification for use in comparing substrate specficities between *D. nigrescens* and *D. cochinchinensis* β-glucosidases, application of *Dalbergia* β-glucosidases to hydrolysis of soy isoflavones, and screening for substrates of β-glucosidases in rice plant and *Dalbergia* seed extracts. Screening of glycosides provided by colleagues in India for hydrolysis by various enzymes was also carried out. Efforts to solve the structures of the isolated glycosides were also carried out, though only the *D. nigrescens* diglycoside substrate structures were fully elucidated.

1.4. Short Description of Methods (ข้อตกลงเบื้องต้น)

Dalbergia cochinchinensis Pierre β-glucosidase was expressed in *Pichia pastoris* and extracted from seeds and purified by column chromatography, as previously described (Srisomsap *et al.*, 1996; Ketudat Cairns *et al.*, 2000; Toonkool et al., 2006). The β-glucosidase from *Dalbergia nigrescens* Kurz, was purified from seeds (Chuankhayan *et al.*, 2005), as well as being expressed in the same system, and purified by dialysis of pichia media, followed by immobilized metal affinity chromatography (IMAC, Chuankhayan et al., 2007b). The β-glucosidases from rice were expressed in *E. coli* strain Origami(DE3) from the pET32 plasmid and purified by IMAC, as previously described (Opassiri et al., 2003, 2006; Chuenchor et al., 2006; Seshadri et al., 2009).

Seeds of *Dalbergia* sp. were collected from trees on the campus of Suranaree University of Technology, Nakhon Ratchasima, Thailand. The seeds were surface sterilized and ground in a blender. The seed coats were separated from the remainder of the seeds (mainly endosperm) and the endosperm portion was extracted in methanol. The seeds were ground further and extracted sequentially with methanol and the crude extracts wre cleared by centrifugation, extracted once with hexane and dried. The extract was redissolved in water back extracted with ethyl acetate, then dried and redissolved in methanol. The extract was further separated by LH20 resin column chromatography, then preparative TLC to give purified *D. nigrescens* glycosides and some semipurified glycosides from *D. cochinchinensis* and *D. nigrescens*.

In this project glycosidase enzymes purified from recombinant expression or natural sources were used to screen for natural glycoside substrates from *Dalberia* sp. seeds and rice seedling shoots and roots, and mature rice flowers, stems and leaves. The plants materials were extracted with methanol or ethanol and the extracts dried. The extract was redissolved in methanol and separated on an LH20 column, then the fractions were digested with or

without the enzymes and the reactions concentrated, then spotted onto analytical TLC plates to compare the with and without enzyme reactions. TLC plates were examined with long and short wavelength UV light for absorbance and fluorescence, then sprayed with 10% sulfuric acid in methanol and heated to 120 °C to detect sugars. Spots that changed positions with the addition of enzymes were considered substrates of the enzyme utilized in the reaction. Purification proceeded with preparative TLC, as desctibed for *D. nigrescens* substrates, and reverse phase high performance liquid chromatography (HPLC), in some cases.

Purified products were analyzed by UV/visible spectrophotometry, mass spectrometry (LCMS), and NMR to determine their structure. NMR techniques used for elucidationof the *D. nigrescens* substrates included ^{1}H-NMR, ^{13}C-NMR, and 2-dimensional analyses: COSY, HMBC, HMQC and DEPT. Attempts were made to crystallize the compounds by concentration and mixing of their alcohol solvents with water and other solvents, although none yielded crystals. Glycoside sugars were also analyzed by hydrolysis with the enzyme and with acid, then separation on TLC under conditions which separate sugars alongside standard sugars for comparison. To determine the linkage cleaved by the *D. nigrescens* β-glucosidase in its natural substrates, the substrate was digested to completion with either *D. nigrescens* β-glucosidase or HCl, and the sugar silylanated with tetramethyl silane and separated on GC-MS with sugar standards.

For analysis of hydrolysis of soy isoflavones by *Dalbergia* enzymes, pure soy isoflavone glycosides, methanolic extracts, and buffered suspensions of soy flour were digested with *D. cochinchinensis* dalcochinase, *D. nigrescens* β-glucosidase or almond β-glucosidase at 30 °C. The soy flour suspensions were separated into soluble and insoluble fractions and extracted with methanol. The amount of glycoside and aglycone remaining were analyzed by silica gel TLC and quantified with reverse phase HPLC.

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

From the work on glycoside characterization, hydrolysis of glycosides by glycosidases, and identification of new glycoside substrates, we were able to contribute to the publication of three papers (Chuankhayan et al., 2005, 2007a, and 2007b), as well as providing data and compounds to contribute to future publications. The identification of the mode of action of *D. nigrescens* β-glucosidase in cutting off a disaccharide from its natural substrates led to a new Enzyme Commission number being designated (E.C. 3.2.1.161). This work also contributed to the training of 2 Ph.D. students and several research assistants. The work suggested that *D. nigrescens* β-glucosidase (and recombinant Dnbglu2) would be appropriate enzymes for application to improving the nutritional value of soy in an industrial setting, though no effort has since been made to do this due to the reluctance of Thai industry to invest in development. In addition three rice glycosides were identified as potential natural substrates for the enzymes in rice, although the small amounts and difficulty of purification have prevented us from determining their structures to date.

2. Materials and Methods

2.1 Materials.

Dalbergia nigrescens Kurz *Dalbergia cochinchinensis* Pierre seeds were collected at Suranaree University of Technology. Rice seeds were obtained from the Nakhon Ratchasima rice seed center, while flowering KDML105 plants were collected at the Pathum Thani Rice Research Center of the Department of Agriculture.

Para-nitrophenyl glycoside substrates, and other commercial glycosides, aside from isoflavone glycosides, were products from Sigma, Fine Chemicals (St Louis, MO, USA), as was almond beta-glucosidase. Diadzein, diadzin, genestein, genestin, malonylgenistin and glycitin were purchased from LC Laboratories (Woburn, MA, USA). Defatted soybean flour from ADM Protein Specialties (Decatur, IL, USA). All other chemicals were analytical grade or better. The IMAC resin 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 by the method of Chuankhayan et al., 2005. The suface-sterilized seeds were ground briefly in a blender and the pericarp removed, then the endosperm was ground to a powder, which was extracted with 3 volumes of absolutemethanol by stirring overnight at room temperature. The extract was filtered and partitioned in a separtory funnel with 1 volume of hexane. The methanol phase was dried by rotary speed vacuum and the residue dissolved with water. The water fraction was extracted with 2.5 volumes of ethyl acetate and then dried by speed vacuum. The dried residue was dissolved with methanol and separated on a 1.2 by 85 cm Sephadex LH-20 gel filtration chromatography column. The compounds were eluted with methanol, and the fractions containing β-glucosidase substrates were identified by digestion with or without β-glucosidase (10% substrate by volume) in 0.1 M NaOAc (pH 5.0) 10 min at 30°C. The products were separated on analytical silica gel 60 F254 aluminum TLC sheets developed twice with solvent A, (EtOAc/methanol/H2O/acetic acid 15:2:1:2 by vol). The sheets were visualized under 254 nm wavelength UV light to identify spots with shifted mobility after enzymatic digest. Dalpatin β-D-apiosyl-6-O-β-D-glucoside (1) and dalnigrecin β-D-apiosyl-6-O-β-D-glucoside (2) were purified from pooled fractions by preparative thin-layer chromatography by developing twice with solvent A. The LH-20 column fractions containing dalpatin β-D-apiosyl-6-O-β-D-glucoside (1) were incubated overnight in 0.1 M NaOAc buffer, pH 5.0, with *D. cochinchinensis* β-glucosidase to hydrolyze low abundance glycoside contaminants before TLC purification of the compound 1 glycoside. The glycosides were digested to the aglycones with 0.1 unit of *D. nigrescens* β-glucosidase by in 0.1 M sodium acetate buffer, pH 5.0, at 30°C, overnight. The aglycone of dalnigrecin was purified by preparative silica gel 60 F254 TLC using solvent B (EtOAc/methanol/H_2O 5:2:1 by vol).

Dalcochinin β-D-glucoside was purified from *D. cochinchinensis* seeds by ethanol extraction. Ten grams of seed powder was stirred 16 h with ethanol at room temperature. The ethanol extract was decanted off the seed powder and filtered, thend dried, 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 Agilent XDB-C_{18} reverse phase column with 38% methanol as solvent on an Agilent 1100 series HPLC with detection by absorbance at 260 nm wavelength on a diode array detector. The dalcochinin glucoside

fractions were collected and dried by speed vacuum. The mass of the purified glycoside was verified to match dalcochinin β-D-glucoside by electrospray mass spectrometry.

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). The compounds were separated with an Eclipse XDBC18 (4.6 by 250 mm, 5 μm) reverse phase column on a Hewlett-Packard series 1100 HPLC (Agilent Corp., Palo Alto, CA) with the UV detection at a wavelength of 260 nm. Solvent A was 0.1% phosphoric acid in water, and solvent B was acetonitrile. The sample was injected in 10% solvent B, and was held at 10% B for 5 min and then increased in a linear gradient from 10 to 35% B over 45 min at a flow rate of 0.8 mL/min.

2.3. Recombinant expression and purification of Dnbglu2

The pPICZα/Dnbglu2 plasmids was linearized with *Sac*I restriction enzyme and then transformed into *P. pastoris* strain YM11430 by electroporation. The transformed *P. pastoris* strain was grown in BMGY medium and induced in BMMY medium with 1% methanol induction as described in the *Pichia* manual (Invitrogen) for small scale expression. The media were tested for hydrolysis of 1 mM *p*NP-Fuc, using 5 μL of media, as described above. 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. To obtain more protein, it was 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). The purified enzyme was used for screening for glycosides from *Dalbergia* seeds, as well as to test for hydrolysis of isoflavone glycosides in soy flour.

2.4. Recombinant expression and purification of rice β-glucosidases

Rice Os3BGlu6, Os3BGlu7 and Os4BGlu12 β-glucosidases were expressed and purified as previously described (Opassiri et al., 2003; 2006; Seshadri et al., 2009). All three enzymes were expressed as N-terminal thioredoxin/hexahistine-tagged fusion proteins from the pET32a (Os3BGlu7) or pET32/DEST (Os4BGlu12 and Os3BGlu6) plasmids. The plasmids were transformed into Origami(DE3) or Origami B(DE3) strains of *E. coli* and expressed by IPTG induction at 20°C, after the culture reached an optical density of 0.4 to 0.6 at 600 nm of growth at 37°C in LB medium containing 50 μg/ml ampicillin, 15 μg/ml kanamycin and 12.5 μg/ml tetracycline. After 16 to 20 h of induction, the cells were pelleted by centrifugation at 4500 rpm for 15 min at 4°C, then frozen at -80°C.

The cell pellets were thawed and suspended in 5 mL/g pellet freshly prepared extraction buffer (20 mM Tris-HCl, pH 8.0, 300 mM sodium chloride, 200 µg/mL lysozyme, 1% Triton-X 100, 1 mM phenylmethylsulfonylfluoride (PMSF), 0.1 mg/ml trypsin inhibitor and 0.25 mg/mL DNaseI). The suspension was incubated at room temperature for 30 min, mixed, then segregated into soluble and insoluble fractions by centrifugation at 12,000 rpm at 4°C for 10 min. The soluble fractions were transferred to a column of immobilized metal (cobalt) affinity chromatography (IMAC) resin (GE Healthcare) that had been washed with 10 volumes of equilibration buffer (20 mM Tris-HCl, pH 8.0, 300 mM NaCl), the column was generally capped and inverted at 4 C for 30 min, before allowing the flow-through fraction to flow out the bottom. The column was then washed with 5 volumes of equilibration buffer, then 5 volumes of 5 mM imidazole in equilibration buffer (wash 1), and finally with 5 volumes of 10 mM imidazole in equilibration buffer (wash 2). The IMAC bound protein was eluted with 250 mM imidazole in equilibration buffer and the fractions tested for activity with 1 mM pNPGlc, and for protein by 12.5% SDS-PAGE. The buffer was then changed and the protein concentrated in a 30 kDa MWCO centrifugal filter (eg. Centricon YM-30, Millipore). Activities were determined by the standard assay described above (1 mM pNPGlc as substrate), and 1 unit of activity was defined as the amount of enzyme that released 1 µmol of *p*NP per min.

For Os3BGlu6, the protein was digested with tobacco etch virus protease (TEV) and further purified by a further round of IMAC. The protein eluted in the equilibrium wash and wash 1 fractions and was concentrated by centrifugal filtration as described above.

2.5. Identification of *Dalbergia nigrescens* natural substrates.

The natural substrates for *Dalbergia nigrescens* had been purified before this work began, but were further characterized. Briefly, the compounds were extracted from the deshelled, powdered endosperm of *D. nigrescens* with methanol, then the extracts were dried, the compounds taken up with water and back-extracted with 2.5 volumes of ethyl acetate, and the water fraction dried. The compounds were redissolved in methanol and separated on an LH20 column and fractions containing substrates were identified by digesting 10 microliter aliquots in the standard enzyme reaction (above) with *D. nigrescens* β-glucosidase. To remove one coeluting compound from compound 1 (Dalpatein apiosyl-glucoside), the pool of the fractions containing this compound was digested with *D. cochinchinensis* β-glucosidase. The substrates were then separated on preparative silica gel TLC (Merck) and the bands scraped off the TLC and extracted from the silica gel with methanol. The substrates were dissolved in deuterated DMSO and analyzed by ^{1}H- and ^{13}C-NMR, along with COSY, NOESY, DEPT, HMBC, and HMQC.

To identify the sugar, 5.5 mgs of each glycoside was boiled with 0.7 mL 0.1 N H_2SO_4 in 1 mL microcentrifuge tube for 25 min. The solution was cooled and extracted with n-butanol, then neutralized with $NaHCO_3$ and the aqueous layer was dried by rotary evaporator. Alternatively, the sugar was removed from the aglycone by overnight digestion with *D. nigrescens* β-glucosidase. The released sugar was isolated by silica gel chromatography, followed by trimethylation or digest with sulfuric acid as described above. The trimethylsilylation of sugar was done by the method of Nikolov and Reilly (1983) with three milligrams of dried residue. The trimethylsilylation mixture was injected into the gas chromatograph and separated with the following conditions: injector at 250 °C; split ratio 20:1; helium carrier gas at 1.2 mL/min; the column temperature was held at 150 °C for 5 min and increased to 190 °C at 20 °C/min and held for 20 min. Mass spectra were collected with a source temperature of 200 °C, a source voltage of 70 eV, and 3.2×10^{-7} torr.

2.6. Identification and purification of rice glycosides

Five to seven-day-old rice seedlings were divided into the root, shoot, and remaining seed for extraction. Rice stems and leaves were cut into approximately 2 cm long pieces. The different fractions were extracted in 2-5 volumes of methanol, stirring overnight, then the methanol extract was separated from the plant material. The extract was centrifuged at 12,000 rpm for 15 min at room temperature to remove any solid material, then extracted with an equal volume of hexane. The methanol fraction was dried on a rotary evaporator. In the case that leaf or stem material with a high amount of chloroform was used, the dried extract was extracted several times in cold acetone until the color faded. The rice extract was redissolved in methanol and aliquots were tested for digestion with enzymes, while the remainder was fractionated on an LH20 liquid chromatography column with methanol as solvent. Fractions were screened by digesting with and without enzymes (10% fraction in a standard enzyme reaction with Os3BGlu7 or Os4BGlu12). Fractions containing apparent glycosides were further fractionated on preparative silica gel TLC. In the case of the mature leaf and stem fractions, the compounds of interest were further separated on an analytical C18 reverse phase HPLC column in 30-40% methanol, 0.05% TFA in water, with UV detection at 220, 254, and 360 nm. Putatively pure peaks were analyzed by NMR and mass spectrometry.

2.7. 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.

2.8. Analysis of *Dalbergia* β-glucosidase hydrolysis of glycosides from soy flour.

Ten grams of defatted soybean flour was extracted with 40 mL of 80% methanol by stirring overnight at room temperature. The solid was removed from the extract supernatant by centrifugation at 12,000 rpm for 15 min. Ten microliters of crude soybean extract was hydrolyzed with 0.001 unit of β-glucosidase in 100 μL 0.1 M sodium acetate buffer, pH 5.5. The reaction mixtures were incubated at 37°C for 10 min or 16 h, and the reaction was stopped by boiling for 5 min. The stopped reactions were dried by speed vacuum and resuspended in 100 μL 10% acetronitrile in 0.1% phosphoric acid/water. A control reaction of crude extract without enzyme was set up in the same manner.

To test the use of *D. nigrescens* β-glucosidse in hydrolysis of glycosides directly in soy flour, the defatted soybean flour was suspended in 0.1 M sodium acetate buffer, pH 5.5, (0.15 g/mL), and 200 μL aliquots of the suspension were incubated with and without 0.001 unit of *D. nigrescens* β-glucosidase. The reaction mixtures were incubated at 37°C for 10 min and the reaction stopped by boiling for 5 min. The reactions were centrifuged at 12,000 rpm for 5 min to remove the supernatant and the solid was extracted with 80% methanol, the methanol extract was removed by centrifugation at 12,000 rpm for 5 min. The reaction supernatant and methanol extract were dried by speed vacuum and resuspended in 20 μL 10% acetronitrile in 0.1% phosphoric acid/water. The hydrolysis of diadzin and genistin in the soy flour suspension was evaluated by thin layer chromatography with butanol: methanol: acetic acid: water (15:1:2:2) (v/v) as solvent.

The kinetic properties of both *Dalbergia* β-glucosidases toward soybean isoflavonoid glycosides were determined by incubating genistin, malonyl genistin and daidzin at

concentrations of 0.035-1 mM in 50 μL reactions containing 5% DMSO in 0.1 M sodium acetate buffer, pH 5.5, with 0.45 ng of *D. nigrescens* or 10 ng of *D. cochinchinensis* β-glucosidase at 30°C. After 10 min, the reactions were stopped by boiling for 5 min. The reactions were dried and resuspended in 50 μL of 0.1% phosphoric acid in water (solvent A), then 20 μL of each sample was injected for HPLC analysis. The column was equilibrated with 85% solvent A. After the sample was injected, ACN was increased from 15% to 35% as a linear gradient over 45 min. The aglycone product of each soybean isoflavonoid glycoside substrate was detected by measuring the absorbance at 260 nm. The amounts of liberated aglycones were calculated from aglycone standard curves of genistein and daidzein over the 0.7-10 mmol range. The calculation of kinetic parameters were obtained from the double-reciprocal (Lineweaver Burke) plots. The kinetic constants shown are mean values of duplicate determination.

2.9. Determination of antioxidant activity (DPPH (diphenylpicryhydrazyl) radical scavenging assay).

The DPPH assay was performed as described by Gerhauser *et al.* (2003). Five microliters of test compounds (in DMSO) were mixed with 195 μL of 100 μM DPPH solution (in ethanol) in a 96-well plate and incubated at room temperature for 30 min. Vitamin C (at 1 mM final concentration) was used as the positive control and the reaction without the test compound was used as the solvent control. Absorbance was measured at 515 nm in a microplate reader. The data was expressed as percent residual DPPH radicals compared with the solvent control, calculated as follows:

$$\% \text{ Radical Scavenging Activity} = \left[1-\left(\frac{\text{Abs}_{\text{DPPH}}-\text{Abs}_{\text{sample}}}{\text{Abs}_{\text{DPPH}}}\right)\right] \times 100\%$$

3. Results

3.1. *Dalbergia nigrescens* natural substrates and the mechanism of their hydrolysis.

Previously, we had purified and characterized two substrates from the seeds of *D. nigrescens*, but the structures of their sugars were still unclear. Although NMR showed signals consistent with β-D-glucopyranosyl and β-D-apiosyl residues, no glucose could be detected to be released from the substrates. However, when the substrate was hydrolyzed with sulfuric acid, glucose and apiose could be identified by TLC and by GC-MS after silylation by comparison to standard sugars. When the enzyme hydrolysis products were compared, a slower migrating compound was detected in place of the sugars, and this compound was hydrolyzed to glucose and apiose by sulfuric acid. Therefore, based on the NMR data (Table 1), the structures were determined to be 6,2′-dimethoxy-4′,5′-methylenedioxyisoflavone 7-O-[β-D-apiofuranosyl-(1→6)-β-D-glucopyranoside] or dalpatein 7-O-β-acuminoside (acuminose being the disaccharide of β-D-apiofuranosyl-(1→6)-D-glucose) and 7-hydroxy-2',4',5',6-tetramethoxyisoflavone 7-O-[β-D-apiofuranosyl-(1→6)-β-D-glucopyranoside], named dalnigrein 7-O-β-D-apiosyl-(1→6)-β-D-glucoside or dalnigrin, as shown in Figure 1. In both cases, it was clear that the sugar must be hydrolyzed from the substrate as a dissacharide (acuminose) by the *D. nigrescens* enzyme.

Since the substrates of *D. nigrescens* β-glucosidase were isoflavonoids, the the isoflavonoid glycosides, its activity toward the soy isoflavonoid glucosides diadzin and genestin were tested, and it was found to hydrolyze them well. When the activity toward these glucosides was compared to the natural substrate diglycosides, it was found that the enzyme hydrolyzed the isoflavone glucosides somewhat better, due to 5-7-fold lower K_m values, though apparent k_{cat} values were similar (Table 2). So, the *D. nigrescens* enzyme could properly be designated as an isoflavone 7-O-β-D-glucosidase.

Subsequent to the initial characterization, the *D. nigrescens Dnbglu2* cDNA was cloned and used to express Dnbglu2 β-glucosidase in *Pichia pastoris*. The recombinant enzyme was tested with the natural substrates that were purified from *D. nigrescens* and *D. cochinchinensis* seeds and the kinetic parameters for hydrolysis determined, as shown in Table 3. Then, the hydrolysis of *Dalbergia* natural substrates was compared with *D. cochinchinesis* dalcochinase, as shown in Figure 2. It was clear that Dnbglu2 hydrolyzed the *D. nigrescens* substrates much better, while dalcochinase hydrolyzed dalcochinin better. Dnbglu2 also hydrolyzed diadzin 3.1-fold and genistin 2.8-fold more rapidly than recombinant dalcochinase expressed in the same system, in terms of relative activity with 1 mM substrate.

3.2. Action of *Dalbergia* β-glucosidases on soy isoflavone glycosides.

To test the usefulness of *Dalbergia* β-glucosidases for conversion of natural glycosides of economic importance, the *D. nigrescens* and *D. cochinchinensis* β-glucosidases were compared with commercial almond β-glucosidase for hydrolysis of isoflavone glycosides in methanolic extracts of defatted soy flour. As seen in Table 4, the *Dalbergia* enzymes hydrolyzed nearly all identified isoflavoniod glycosides faster than almond b-glucosidase, except that *D. cochinchinensis* dalcochinase hydrolyzed glycitin at nearly the same rate as almond β-glucosidase. However, *D. nigrescens* exhibited the most rapid hydrolysis of all substrates, including diadzin, genistin, glycitin, acetyl diadzin, malonyl

diadzin, acetyl genistin and malonyl genistin, the last of which was not significantly hydrolyzed by almond β-glucosidase after 16 h of incubation.

Table 1. ^{1}H and ^{13}C NMR Spectral Data of Compounds 1, 2 and 2a.

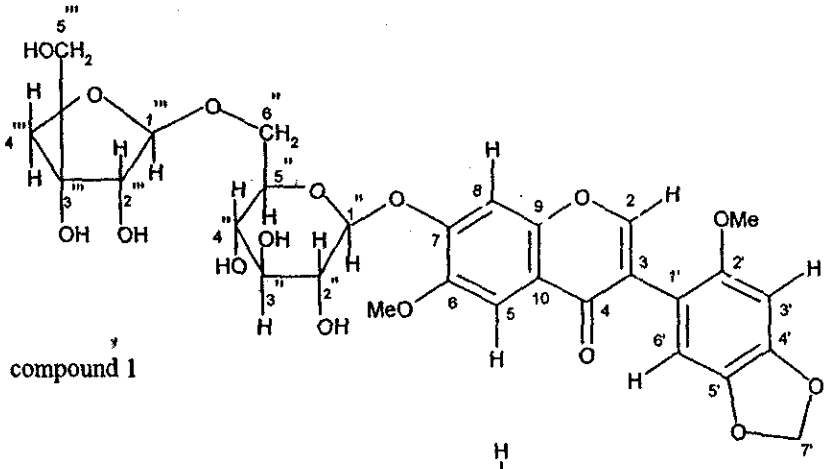
position	Compound 1(DMSO-d_6)			Compound 2 (D_2O)			Compound 2a (CD_3OD)		
	^{13}C	^{1}H	HMBC	^{13}C	^{1}H	HMBC	^{13}C	^{1}H	HMBC
2	154.42 CH	8.19 (s)		156.40 CH	8.03 (s)		156.24 CH	7.87 (s)	
3	121.03 C		2, 3′, 6′	121.05 C		2, 3′, 6′	120.52 C		2, 3′, 6′
4	174.66 C		2, 5, 8	177.47 C		2, 5, 8	176.48 C		2, 5, 8
5	104.63 CH	7.44 (s)	8	105.21 CH	7.17(s)	8	102.72 CH	6.50 (s)	8
6	147.31 C		5, 8, OMe	151.43 C		5, 8, OMe	152.92 C		5, 8, OMe
7	151.41 C		5, 8	152.29 C		5, 8	153.30 C		5, 8
8	103.72 CH	7.32 (s)	5	104.10 CH	7.35 (s)	5	101.97 CH	7.33 (s)	5
9	151.19 C		2, 5, 8	147.58 C		2, 5, 8	150.18 C		2, 5, 8
10	117.68 C		2, 5, 8	111.92 C		2, 5, 8	111.02 C		2, 5, 8
1′	112.92 C		2, 3′, 6′	115.60 C		2, 3′, 6′	113.95 C		2, 3′, 6′
2′	152.77 C		3′, 6′, OMe	152.16 C		3′, 6′, OMe	153.30 C		3′, 6′, OMe
3′	95.45 CH	6.87 (s)	6′	98.72 CH	6.71 (s)	6′	98.76 CH	6.73 (s)	6′
4′	147.79 C		3′, 6′, 7′	149.75 C		3′, 6′,OMe	152.06 C		3′, 6′,OMe
5′	140.26 C		3′, 6′, 7′	142.35 C		3′, 6′,OMe	143.10 CH		3′, 6′,OMe
6′	110.90 CH	6.83 (s)	3′	118.11 CH	6.86(s)	3′	116.90 CH	6.93 (s)	3′
7′	101.08 CH	6.0 (s)							
1″	99.90 CH	5.08(d) J=6.84		100.26 CH	5.21(d) J=7.00				
2″	72.98 CH	3.33		72.94 CH	3.65				
3″	76.67 CH	3.34		77.09 CH	3.86				
4″	69.80 CH	3.15		69.57 CH	3.59				
5″	75.65 CH	3.60		75.41 CH	3.76				
6″	67.85 CH_2	3.88(d) J=9.19		67.76 CH_2	4.02(d) J=9.15				
1‴	109.48 CH	4.80(d) J=2.94		109.38 CH	5.02(d) J=2.87				
2‴	75.85 CH	3.77		75.69 CH	3.69				
3‴	78.60 C			79.51 C					
4‴	73.30 CH_2	3.93		73.87 CH_2	3.92				
5‴	63.32 CH_2	3.37		64.02 CH_2	3.53				
6-OMe	55.75	3.93		6-OMe 56.67	3.73		6-OMe 55.98	3.80	
2′-OMe	56.63	3.66		2′-OMe 56.85	3.68		2′-OMe 56.35	3.75	
				4′-OMe 56.17	3.85		4′-OMe 54.68	3.95	
				5′-OMe 56.38	3.82		5′-OMe 55.65	3.90	

Note: Compound one corresponds to *D. nigrescens* substrate DnS1 or Dalgrecin (dalpatein apiosylglucoside), while Compound 2 is substrate DnS2 or Dalnigrin and Compound 2a is its aglycone.

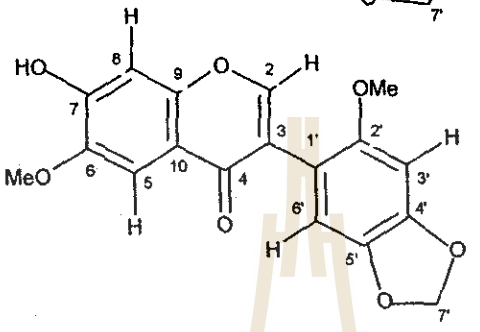
Table 2. Kinetics of hydrolysis of natural substrates and soy isoflavone glucosides

Substrate	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($M^{-1} s^{-1}$)
pNP-β-D-glucoside	14.7	10.4	876
pNP-β-D-fucoside	1.8	7.0	4020
Dalnigrecin (DnS1)	0.5	465	9.9×10^5
Dalnigrin (DnS2)	0.7	334	4.4×10^5
Daidzin	0.11	480	4.4×10^6
Genistin	0.09	500	5.6×10^6

The values of kcat and k_{cat}/K_m were estimated assuming a subunit molecular weight of 62 kDa.

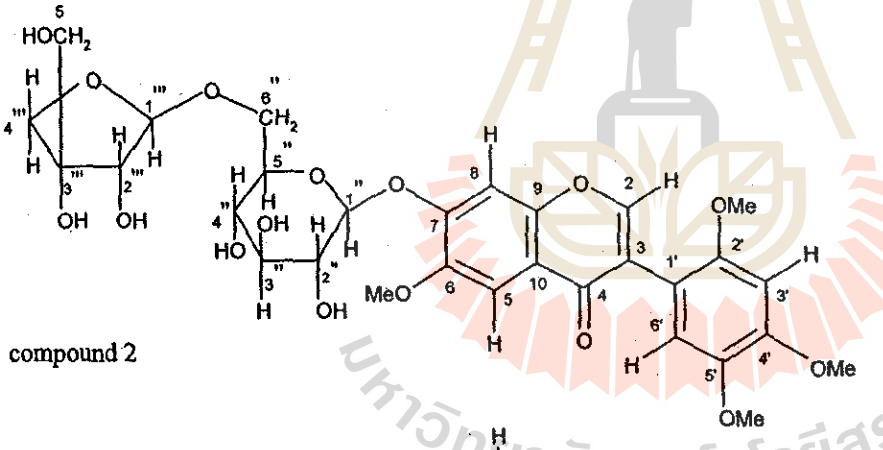


compound 1

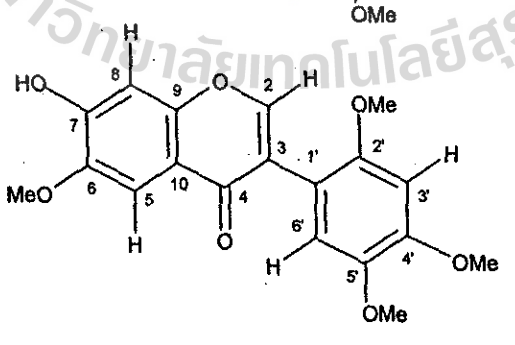


compound 1 a

Compound 1



compound 2



compound 2 a

Compound 2

Figure 1. Structures of *D. nigrescens* natural substrate glycosides and their aglycones. Compound 1 corresponds to Dalnigrecin (dalpatein β-apiosyl glucoside), while Compound 2 corresponds to Dalnigrin (dalnigrein β-apiosyl glucoside, which are also designated D. nigrescens β-glucosidase substrates DnS1 and DnS2, respectively.

Table 3. Kinetic properties of *D. nigrescens* β-glycosidase toward its natural substrates.

Substrates	Dnbglu2		
	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($s^{-1}M^{-1}$)
dalpatein-7-O-β-D-apiofuranosyl (1,6)-β-D-glucopyranoside	5.1±3.5	27 ± 15	(5.3×± 3.1) ×10^3
dalnigrein-7-O-β-D-apiofuranosyl (1,6)-β-D-glucopyranoside	4.25 ± 0.90	239 ± 97	(5.6 ± 2.3) ×10^4
dalcochinin-β-D-glucopyranoside	7.4 ±1.8	62 ± 13	(8.4 ± 1.8) ×10^3

*Calculation of kinetic parameters with standard errors were obtained from linear regression of the double-reciprocal (Lineweaver–Burk) plots with the Enzfitter 1.05 program (Elsevier Biosoft, Cambridge, U.K.).

Figure 2. TLC of dalcochinin β-glucoside, dalpatein apiosylglucoside (DnS1) and dalnigrein apiosylglucoside (DnS2) hydrolysis by recombinant Dnbglu2 and *D. cochinchinensis* β-glucosidases. 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, dalcochinin std; lane 5, dalcochinin hydrolyzed with recombinant *D. cochinchinensis*; lane 6, dalcochinin hydrolyzed with Dnbglu2; lane 7, DnS1 std; lane 8, DnS1 hydrolyzed with recombinant *D. cochinchinensis*; lane 9, DnS1 hydrolyzed with Dnbglu2; lane 10, DnS2 std; lane 11, DnS2 hydrolyzed with recombinant *D. cochinchinensis*; lane 12, DnS2 hydrolyzed with Dnbglu2.

Table 4. Comparison of the effect of prolonged incubation of *Dalbergia* β-glucosidases and almond β-glucosidase. The crude extract was hydrolyzed with 0.001 unit of the β-glucosidases for 10 min and 16 h at 37°C.

Soybean glycosides	% hydrolysis of substrates in extract				
	D. nigrescens β-glucosidase		*D. cochinchinensis* β-glucosidase		Almond β-glucosidase
	10 min	16 h	10 min	16 h	16 h
Daidzin	100	100	52	100	74
Glycitin	100	100	5	31	33
Genistin	100	100	62	98	70
Malonyldaidzin	5	60	0	10	10
Acetyldaidzin	100	100	0	44	17
Malonylgenistin	63	96	0	12	0
Acetylgenistin	100	100	0	47	18

To further characterize the differences between the two *Dalbergia* enzymes, their kinetic parameters for hydrolysis of diadzin, genistin and malonyl genistin were determined. As shown in Table 5, *D. nigrescens* hydrolyzed diadzin and genistin with 6-fold lower Km values than *D. cochinchinensis*, and also showed a nearly 10-fold higher k_{cat} for diadzin, although its k_{cat} for genistin ws slightly lower. In contrast, *D. cochinchinensis* had a much lower K_m for malonyl genistin, but barely hydrolyzed it, due to its extremely low k_{cat}, which suggests it can bind mainly in an unproductive or suboptimal position for hydrolysis. In contrast, *D. nigrescens* had a >200-fold higher k_{cat}, with a 5-fold higher K_m, thereby allowing it to hydrolyze genistin more rapidly at nearly all concentrations.

Table 5. Kinetic properties of purified *Dalbergia* β-glucosidases with soy isoflavone glycosides.

	D. nigrescens β-glucosidase			*D. cochinchinensis* β-glucosidase		
substrate	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($M^{-1}s^{-1}$)	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($M^{-1}s^{-1}$)
Genistin	0.06 ± 0.007	700 ± 40	$11 \times 10^6 \pm 7 \times 10^5$	0.38 ± 0.026	816 ± 53.8	$4.3 \times 10^6 \pm 5.0 \times 10^4$
Daidzin	0.14 ± 0.017	298 ± 18	$2.1 \times 10^6 \pm 1.3 \times 10^5$	0.88 ± 0.007	31 ± 2.51	$3.7 \times 10^4 \pm 1.4 \times 10^2$
Malonyl genistin	0.48 ± 0.042	52 ± 9	$1.1 \times 10^5 \pm 2.2 \times 10^3$	0.08 ± 0.008	0.23 ± 0.015	$2.9 \times 10^3 \pm 146$

Although *D. nigrescens* exhibited high activity toward isoflavone glycosides in methanolic extracts, it was unclear how useful this would be for treatment of soy flour to increase the levels of free isoflavones. So, D. nigrescens enzyme was tested for treatment of soy flour suspended in buffer. As seen in Figure 3, treatment with *D. nigrescens* β-glucosidase not only resulted in hydrolysis of diadzin and genistin in the aqueous phase, but also of the diadzin and genistin that remained associated with the flour particles and could be extracted from these particles with methanol. Thus, it appears to be applicable in conditions that could be used in food processing.

Many of the desireable properties of diadzin and genistin stem from their antioxidant capabilities, so the ability of *D. nigrescens* β-glucosidase to release antioxidant activity was assessed. The antioxidant properties of daidzin and genistin were tested with and without treatment with *D. nigrescens* β-glucosidase by the DPPH assay with daidzein and genistein as positive controls. After hydrolysis with *D. nigrescens* β-glycosidase, the antioxidant activity of 1 mM daidzin and genistin were the same as 1mM daidzein and genistein, with approximately 6% and 30% of the radicals scavenged, respectively. Without enzyme hydrolysis, no radicals scavenging activity could be detected for daidzin and genistin at the same concentration. However, crude soy flour extract with or without digestion was found to have 100% scavenging activity in this assay, suggesting that compounds other than these isoflavones were the main antioxidants in this extract.

Although several other spots that changed position when treated with *D. cochinchinensis* or *D. nigrescens* β-glucosidases were identified in the seeds from which these enzymes were derived, none of these compounds could be purified to homogeneity. It appeared that these compounds were at lower concentrations and tended to be mixed with other glycosides, making their purification difficult. In addition, we were told that the group of Dr. Palangpol Kongsaeree of Mahidol University had purified several of these compounds from *D. cochinchinensis* seed extracts, so we did not pursue these compunds further.

3.3. Rice Glycoside Screening.

Since we have identified the genes for 34 putative GH1 β-glucosidases in rice (Opassiri et al., 2006), it was of interest to see if natural substrate glycosides could be determined for these enzymes, which might give a view of their function, and yield insights into the roles of β-glucosidases in plants. Therefore, we started to screen for rice glycosides that could be hydrolyzed by rice enzymes from rice extracts, similar to the identification of substrates from Dalbergia seeds, with the Os3Bglu7 (BGlu1) and Os4BGlu12 GH1 b-glucosidases and a GH family 5 enzyme, designated GH5BG, that had been expressed in a recombinant *E. coli* system in our group. Initial work was conducted with all three enzymes, but later mainly the Os4BGlu12 was used for screening, since it was relatively stable and one batch of purified enzyme could last longer than the other enzymes. Initial screening of the LH20 column of a methanolic extract of 5-day-old rice seedling shoots with Os3BGlu7 and Os4BGlu12 is shown in Figure 3.

The bright white fluorescent substrate that was identified by TLC was further investigated by HPLC on a C8 reverse phase column. When the pool of the fractions containing that spot was injected onto the HPLC with and without enzymatic digest, several new peaks appeared in the digested pool compared to the undigested sample, but the glycoside peaks that were being digested were not obvious in the undigested sample chromatogram (Figure 4). Nonetheless, the fractions containing the glycoside could be identified by running the fractions collected from the HPLC on TLC. One spot was collected and purified by this method, as shown in Figure 5. The nearly purified sample was analyzed by NMR, but the amount of material was less than 1 mg and the 300 MHz NMR was not able to give a clear spectrum from this small amount of incompletely purified material. Since the postdoctoral researcher conducting this extraction went to a new position, the purification of this compound was not continued. It was noted that fluorescence is generally very sensitive, so spots identified by fluorescence may not have sufficient material for characterization (despite the fact that the fluorescent substrates of *D. nigrescens* could be purified and characterized).

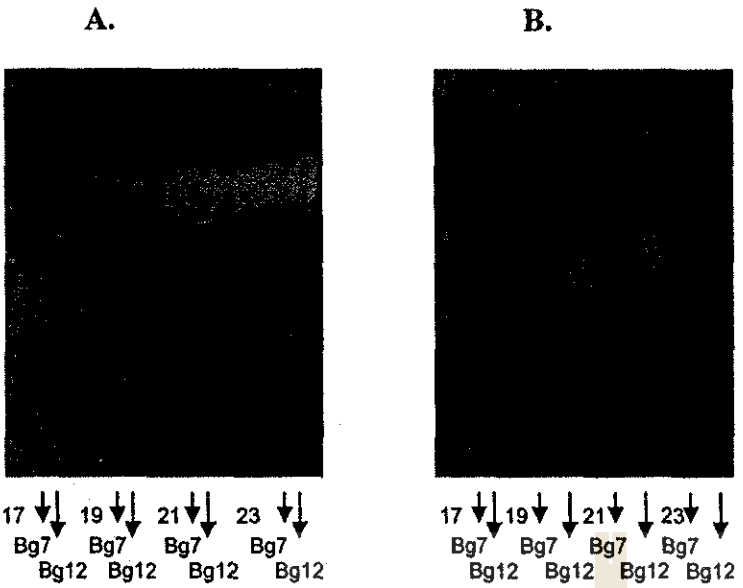


Figure 3. TLC of undigested and digested LH20 fractions of a 5-day-old rice seedling shoot methanolic extract. A. 10 min digest of LH20 fractions 17, 19, 21 and 23 alone and with the Os3BGlu7 (Bg7) and Os4BGlu12 (Bg12) β-glucosidases. B. Overnight digest of the same fractions with the same enzymes (with TLC developed a shorter time). The TLC were visualized under UV light. Note the bright fluorescent white spot in fractions 19-23 is decreased after incubation with either of the two enzymes.

Figure 4. Analytical C8 HPLC of pooled LH20 fractions containing the fluorescent glycoside before (A) and after (B) digest with Os4BGlu12 β-glucosidase. The HPLC elution gradient was in water for 5 min, then 0 to 60% methanol over 20 min, then from 60 to 100% methanol in 5 min, and continuing in 100% methanol for 5 min, before returning to water. Absorbance was monitored at 220 nm (UV).

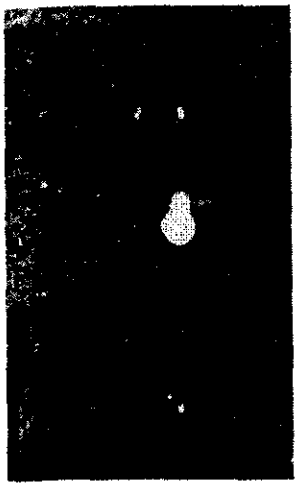


Figure 5. TLC of Semipreparative C8 HPLC fractions of the LH20 fractions 19-25 pool of 5-day-old seedling shoot methanol extract. Detection under UV light was used to give the fluorescent spot and spots that adsorbed the background (F254) fluorescence. The white spot seen in the Figure 3 fractions can be seen to resolve into two closely linked spots in fractions 12 and 13.

Further work was done to test different rice seedling and flowering stage tissues. Glycosides were also identified in seedling roots, but the complexity of the sample and the small amounts also made these compounds difficult to purify. However a set of glycosides was identified in fractions of rice leaves and stem from flowering stage, which were purified, as described for the seedling shoot putative glycoside. The TLC spot position from initial screening was used to identify the fractions containing the same compounds in large scale LH20 column chromatography runs, as indicated in Figure 6. Once these fractions were identified and pooled, they were separated by preparative silica gel TLC to generate a more highly purified fraction, which was injected onto C18 HPLC to separate its components. Since these fractions had quite similar polarity, the HPLC conditions had to be adapted to get the maximal separation. Then, at least three glycoside components, designated Cl/sD1, Cl/sD2 and Cl/sD3, could be resolved as shown in Figure 7, with a fourth impure glycoside fraction eluting in front of these resolved peaks at 6-7 min. Repeated analytical HPLC runs were used to resolve the peaks, since attempts to move to semipreparative columns failed to resolve the peak components. These compounds were further analyzed by mass spectrometry and proton NMR were run on the isolated components, but the signals were weak and unclear. The mass spectrometry (Figure 8) indicated apparent molecular masses of 492 (M+H = 493) for Cl/sD1 and 688 (M+H = 389) for both Cl/sD2 and Cl/sD3.

To test whether other rice β-glucosidases hydrolyze the same glycosides, hydrolysis of the HPLC peak compounds by Os3BGlu6 and Os3BGlu7 was compared to that by Os4BGlu12. As shown in Figure 9, all three enzymes seemed to rapidly hydrolyze Cl/sD1, and no obvious differences could be seen in their rates of hydrolysis of this compound. However, Os3BGlu6 hydrolyzed Cl/sD2 most rapidly, while Cl/sD3 was hydrolyzed most rapidly by Os4BGlu12. This later result is interesting in that the two compounds have identical molecular masses, suggesting they may be isomeric compounds, but the three rice enzymes tested could distinguish them. However, all three enzymes could hydrolyze each of these glycosides to some extent. In contrast, the impure components that eluted at 4 min seemed to have only a small amount of hydrolysis (light band at the top of the TLC) by all

three enzymes, while the 6 min peak was only significantly hydrolyzed by Os4BGlu12. These last two peaks are not yet pure enough and have not been further analyzed.

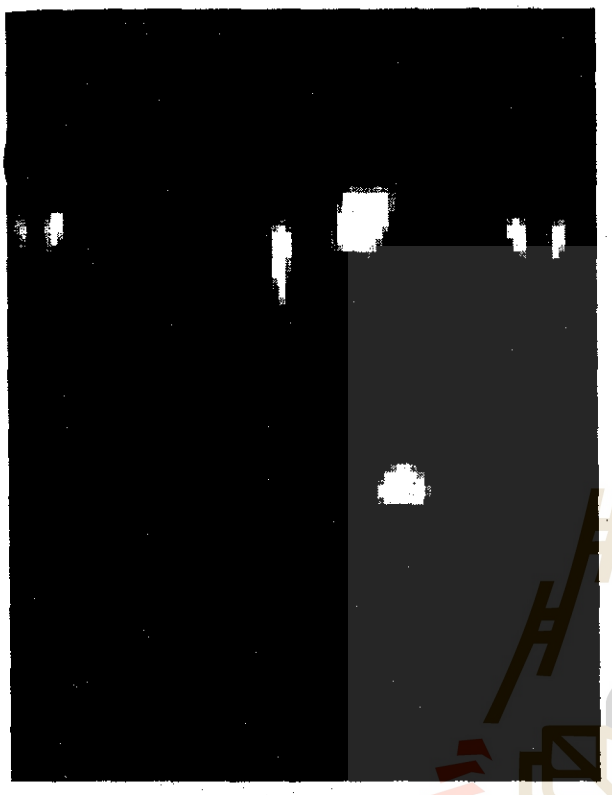


Figure 6. Fractions from the LH20 column of a methanolic extract of mature rice leaves and stems, with the spot containing glycoside indicated by the arrow. Note that fractions from a previous run identifying the glycoside by its hydrolysis are run on the sides to indicate the position of the glycoside spot.

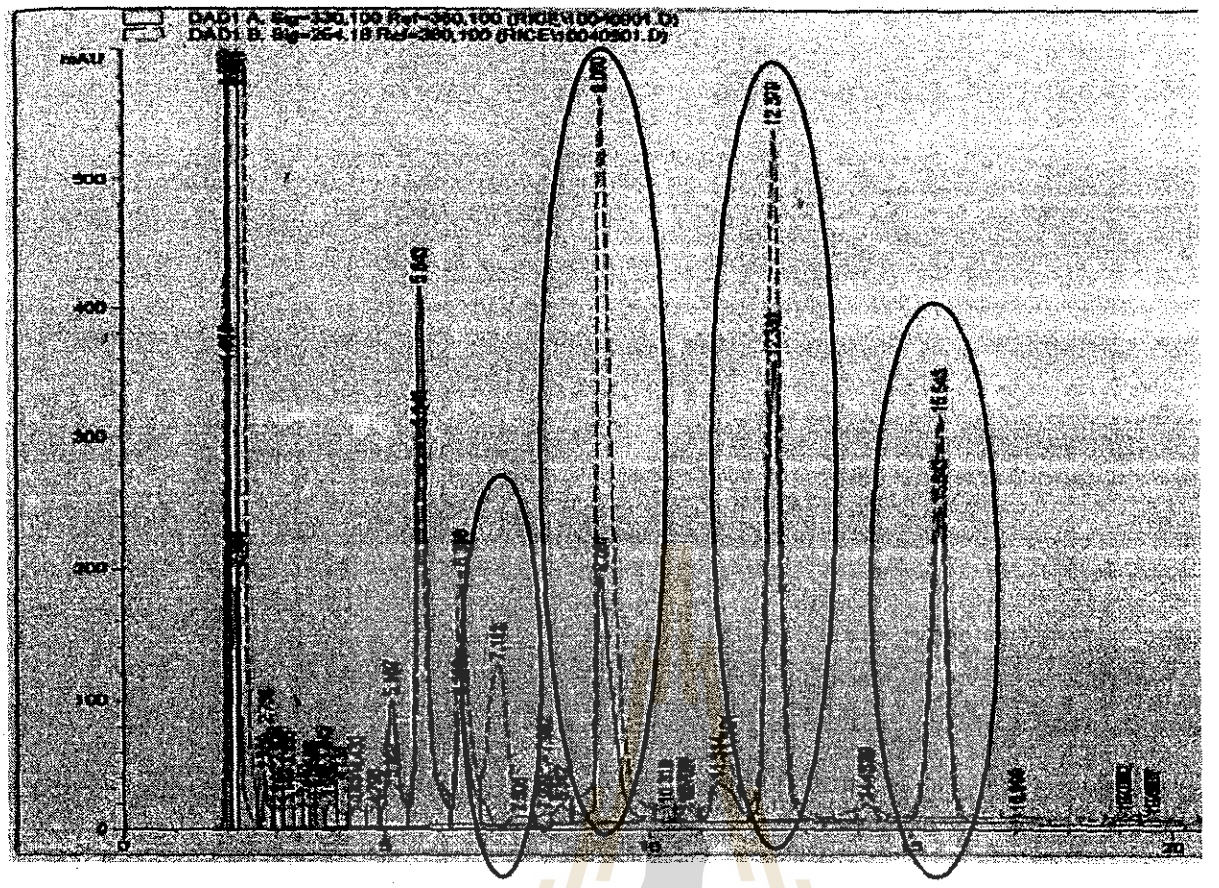


Figure 7. Separation of the Cl/sD components from LH20 and preparative TLC on reverse phase HPLC. The peaks confirmed to contain glycosides are circled, starting from Peak 6, followed by Cl/sD1, Cl/sD2 and Cl/sD3. The peaks were separated by isocratic elution on a C18 analytical column in 30% methanol in 0.1% TFA with detection with 254 and 330 nm light.

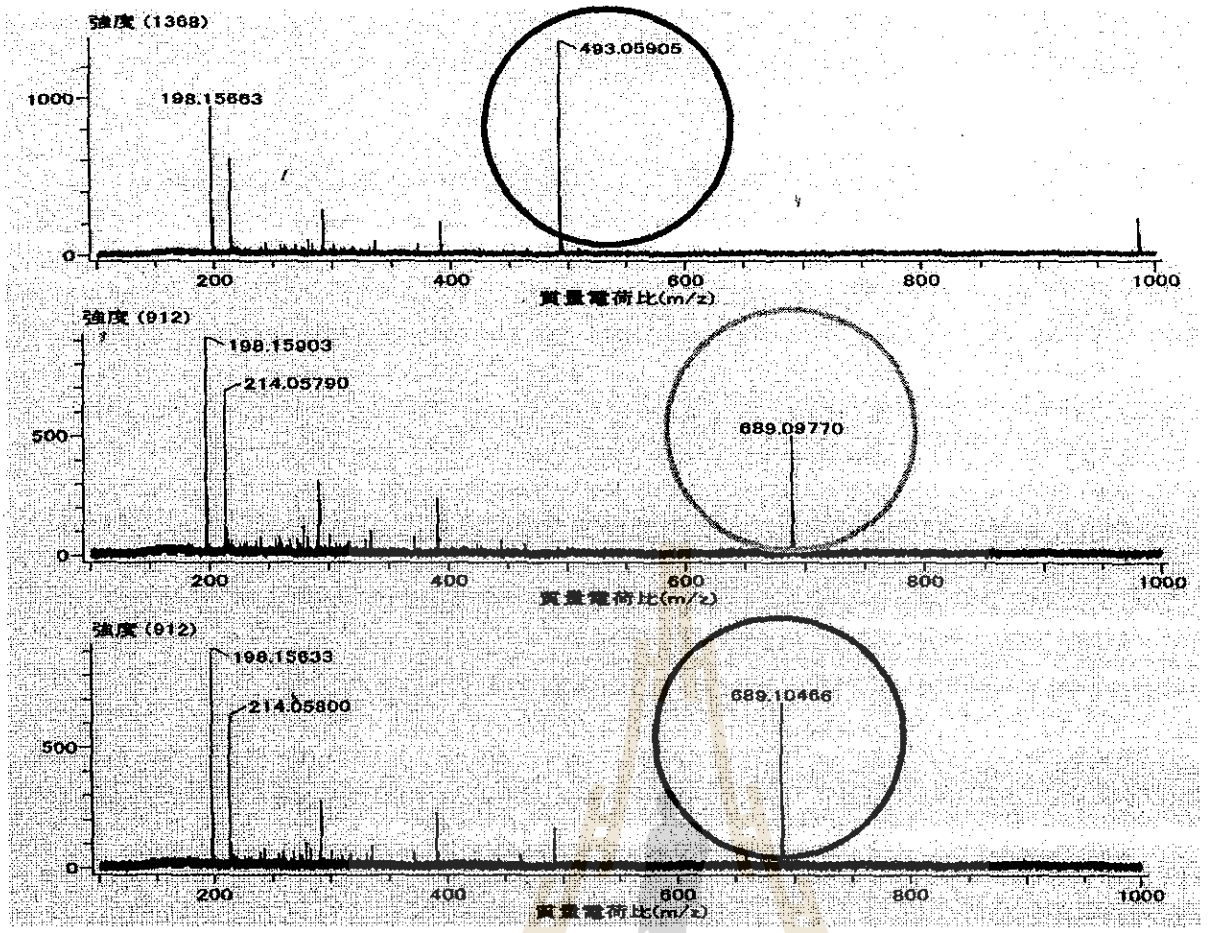


Figure 8. Mass spectra of Cl/sD1 (top), Cl/sD2 (middle) and Cl/sD3 (bottom). The mass spectra were collected by electrospray ionization mass spectrometry in positive ion mode.

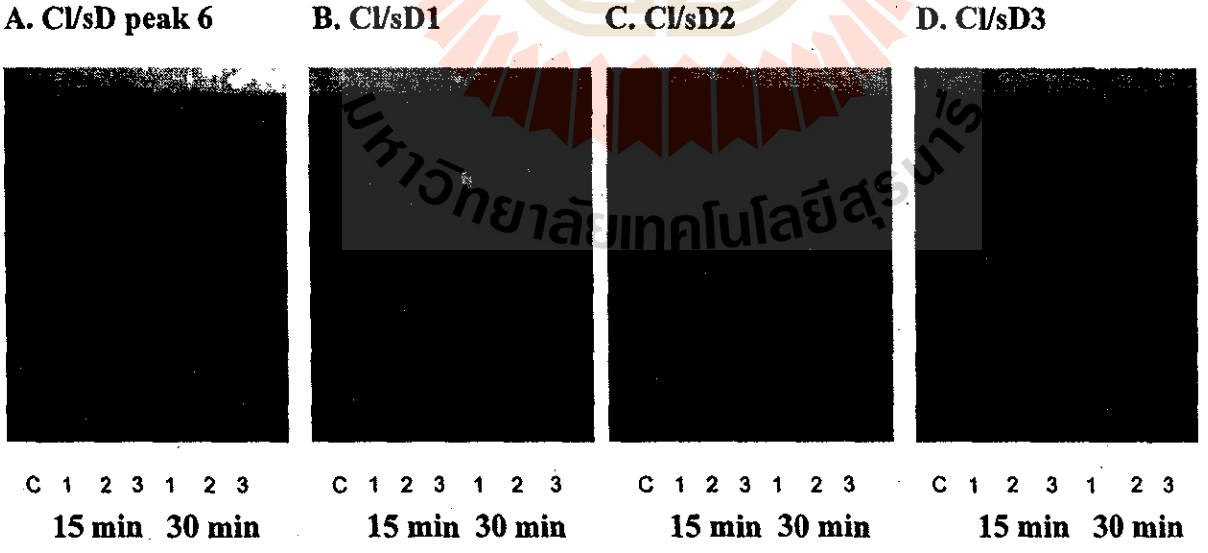


Figure 9. TLC of Cl/sD peak fractions digest by Os4BGlu12, Os3BGlu6 and Os3BGlu7. The fractions were run as a control reaction (C), reactions with Os4BGlu12 (1), Os3BGlu6 (2) and Os3BGlu7 (3). A. is Cl/sD HPLC separation peak 6, B is Cl/sD1, C is Cl/sD2 and D is Cl/sD3. The digests included 1 mg/mL compound, 1 mg enzyme in 50 mM sodium acetate, pH 5.0. The reactions were separated on Silica gel F254 analytical TLC and detected by fluorescence absorption under UV light.

Section 4 Analysis

4.1 Discussion:

This project consisted of two components, 1) characterization of *Dalbergia nigrescens* β-glucosidase natural substrates and hydrolysis of these and other known glycosides of interest, such as soy flour isoflavones, and 2) identification of novel glycosides based on their hydrolysis by the β-glucosidases available in our laboratory. Initially, these parts would have both included *Dalbergia* β-glucosidases, but the latter part of the project focused on rice β-glucosidases at the end, due to the fact that we would like to determine the roles of the various β-glucosidases found in rice. This work supplemented other projects in the laboratory and contributed to three *D. nigrescens* β-glucosidase papers (Chuankhayan et al., 2005, 2007a, 2007b), though these projects were largely funded from other sources. The rice glycoside work is ongoing, and it is hoped that it will eventually yield publications as well.

When we started this project, we had run many of the NMR analyses of the *D. nigrescens* substrates, but we were still not able to complete the structures, due to the unexpected lack of glucose release upon hydrolysis. However, the discovery that the *D. nigrescens* enzyme could release the sugar as a disaccharide (β-D-apiofuranosyl-(1,6)-D-glucose, also called acuminose) solved this inconsistency and allowed resolution of the structures of dalpatein apiosylglucoside and dalnigrin (dalnigrein apiosyl glucoside), as shown in Figure 1. In fact, it was previously noted that chick pea β-glucosidases could release acuminose from isoflavones (Hösel and Barz, 1975), and furcatin hydrolase, which is primarily a disaccharidase releases the same sugar (Ahn et al., 2004), although furcatin is not an isoflavonoid. However, the reinvestigation of this activity led to a new enzyme commission number being assigned to it, E.C. 3.2.1.168. This type of disaccharidase activity can only work for (1,6)-linked disaccharide, since the nonreducing sugar must be folded back alongside the aglycone in the active site, since the bottom of the pocket has room for only glucose (Chuankhayan et al., 2007b). The enzyme cannot hydrolyze β-1,4-linked oligosaccharides and glycosides, such as cellobiose or pNP-cellobioside, since they are straight chains that cannot fold back on themselves like the β-(1,6)-linkage can.

As noted in the Introduction, there has been significant interest in the release of isoflavones from their glycosides, due to their bioactivity and the possible increase in bioavailability of these compounds. We also thought that the presence of more free isoflavones could provide additional antioxidant activity to prevent oxidation of the soy products leading to loss of quality. When tested, it was found that *D. nigrescens* had very good ability to release soy isoflavones from their glycosides, including 6-O-modified glucosides, such as acetyl-genestin and malonyl genestin. These activities correlate well with the natural substrates, which contain the addition of an apiosyl group on the glucose 6 carbon. Recently a isoflavone β-glucosidase from soybean roots has been identified that had even higher specificity for malonyl genistin (Suzuki et al., 2006). Nonetheless, the general ability of *D. nigrescens* to hydrolyze all soy isoflavone glucosides is useful for processing of soy products. The demonstration that *D. nigrescnes* β-glucosidase can hydrolyze even the soy isoflavones associated with the flour particles shows this to be the case. However, the use of this reaction for increasing the antioxidant content of the soy flour in order to decrease oxidation did not appear useful, since the overall antioxidant content in the methanol extract of the flour was not increased by treatment with the enzyme.

Initial studies with *D. cochinchinensis* and *D. nigrescens* seed extracts indicated that there were additional glycoside substrates for these enzymes in their seeds. However, the

amounts were evidently lower than the glycosides substrates that had been purifed, dalcochinin for *D. cochinchinesis* and dalpatein apiosylglucoside and dalnigrin for *D. nigrescens*. As our colleagues in Mahidol University had already purified some of the *D. cochinchinesis* compounds and the *D. nigrescens* glycosides appeared harder to purify, no further progress was made in these glycosides. Instead, we turned our attention to rice β-glucosidase glycoside substrates, a work we had begun on a previous grant, but had only completed initial screenings.

The rice β-glucosidases that we used to screen for glycosides, Os4BGlu12 and Os3BGlu7 had previously been shown to act on oligosaccharides, but had lower activity on some natural glycosides, suggesting they may have alternative substrates in the rice plant (Opassiri et al., 2003, 2004, 2006). In fact, these two enzymes were able to hydrolyze glycosides in each of the extracts, but the change was difficult to see without fractionation first to decrease the complexity. The first purification from rice shoots showed that both enzymes hydrolyzed the same fluorescent compound. Since Os4BGlu12 is more stable for storage, it was used for further screening. The initial fluorescent compound could not be purified in sufficient quantity and purity to characterize the structure, but served to prove the concept. Later, we found compounds from seedling root and mature plant leaf and stem. The leaf and stem compound was further fractionated by preparative TLC and HPLC, which showed there were still several compounds. However, three HPLC peaks appeared to have relatively pure compounds Cl/sD1, Cl/sD2, and Cl/sD3, the masses of which could be determined to be 492, 688 and 688 amu, respectively. The first compound mass correlates to the mass of several flavonoid glycosides and other glycosides, including Crassifoside C, Tarenninoside B, Crassifoside F, Licoagroside A, Isothymusin-8-O-β-D-glucoside, Yixigensin, Camaraside, Coccinoside A, Lagotiside, Andrographidin B, Eupalitin 3-O-β-D-glucoside, Quercetin-5,3'-dimethyl ether-3-glucoside, Tricin 4'-O-glucoside, Tricin 5-O-β-D-glucoside, Tricin 7-O-β-D-glucoside, 3',7-Dimethoxyhyperin, Homotectoridin, Cirsiliol 4'-glucoside, 4',5,7-Trihydroxy-3,6-dimethoxyflavone 7-O-β-D-glucoside, Jaceoside, Prudomenin, and Glucotricin. Of these tricin 4'-O-glucoside and 7-O-β-D-glucoside have been reported in rice, where they were found to be probing stimulants for white plant hopper (Adjei-Afriyie et al., 2000). So, it is possible that Cl/sD1 is one of the tricin glucosides or another glycoside of a trihydroxy, dimethoxy flavone. Further work will be needed to prepare enough enzyme for reliable NMR analysis.

4.2. Conclusions and Comments

Although the rice compounds have yet to have their structures identified, this project has successfully aided in the identification of these substrates, as well as aiding in the characterization of *D. nigrescens* β-glucosidase hydrolysis of natural glycosides from its own seeds and soybeans. As such, it has contributed to the publication of three international papers (Chuankhayan et al., 2005, 2007a, 2007b), as well as the training of several students. The rice compounds that have been identified will be characterized completely in the near future, and they may then be used for enzymatic studies to see what is the kinetic basis for the more rapid hydrolysis of Cl/sD2 by Os3BGlu6 and Cl/sD3 by Os4BGlu12. If they are good substrates, we will also look at their interactions with these enzymes by protein crystallography, since the structures of these enzymes have been solved.

In fact, the content of the project had to be adapted to adjust to the lower than requested budgets and availability and abilities of students and assistants, but this is not unusual. Overall, much was achieved in this project.

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Curriculum Vitae

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

Name: James Last name: Ketudat-Cairns

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

3. ตำแหน่งปัจจุบัน .....รองศาสตราจารย์. .....

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: Structural studies of rice β-glycosidases. November, 2006-May, 2009. The National Synchrotron Research Center. (1.8 million baht). 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. Principle investigator: Isolation and Characterization of Rice β-glucosidase cDNAs

Sept. 2000-August, 2002 Thailand Research Fund BGJ Grant (300,000 baht).

6. Principle Investigator: Isolation and characterization of Glycosyl Hydrolases from Thai Plants. Sept. 1996 – Aug., 1999. (Thai Research Fund Grant RSA 011/2539)
7. Coinvestigator: Cloning and sequence anaylsis of the β-glucosidase/β-fucosidase enzyme from Thai Rosewood. Paper accepted for publication, J. Biochem. Dec. 2000.
8. Coinvestigator: Modeling of the disulfide knot in protein structure. Project not published.
9. 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: Protein structure-function relationships in plant β-glucosidases II. BRG5080007. Sept., 2007-August, 2010. The Thailand Research Fund (2,000,000 baht).
2. Coinvestigator: Abnormalities in proteins and enzymes in relation to genetic diseases found in Thailand. (Chulabhorn Research Institute grant)

Publications (Journal Articles)

1. Seshadri S, Akiyama T, Opassiri R, Kuaprasert B, and Ketudat Cairns J. 2009. Structural and enzymatic characterization of Os3BGlu6, a rice β-glucosidase hydrolyzing hydrophobic glycosides and (1→3)- and (1→2)-linked disaccharides *Plant Physiology*; **151,** 47-58. doi:10.1104/pp.109.139436 (ISI IF 6.110, 2008).

2. Akiyama T, Jin S, Yoshida M, Hoshino T, Opassiri R, Ketudat Cairns JR. 2009. Expression of an endo-(1,3;1,4)-beta-glucanase in response to wounding, methyl jasmonate, abscisic acid and ethephon in rice seedlings. *Journal of Plant Physiology* 2009 Jun 29. [Epub ahead of print] (ISI IF 2.437, 2008)

3. Chuenchor W, Pengthaisong S, Robinson RC, Yuvaniyama J, Oonanant W, Bevan DR, Esen A, Chen C-J, Opassiri R, Svasti J, Ketudat Cairns JR. 2008. Structural insights into rice BGlu1 β-glucosidase oligosaccharide hydrolysis and transglycosylation. *Journal of Molecular Biology* **377** (4), 1200-1215. doi: 10.1016/j.jmb.2008.01.076 (ISI IF 4.146, 2008).

4. Keeratichamroen S, Ketudat Cairns JR, Wattanasirichaigoon D, Wasant P, Ngiwsara L, Suwannarat P, Pangkanon S, Tanpaiboon P, Rujirawat T, Svasti J. 2008. Molecular analysis of the iduronate-2-sulfatase gene in Thai patients with Hunter syndrome. *Journal of Inherited Metabolic Disease* doi:10.1007/s10545-008-0876-z (ISI IF 1.574, 2006)

5. Tantanuch W, Chantarangsee M, Maneesan J, Ketudat Cairns JR. 2008. Genomic and expression analysis of glycosyl hydrolase family 35 genes from rice (*Oryza sativa* L.). *BMC Plant Biology* **8**(1), 84. doi:10.1186/1471-2229-8-84 (ISI IF 4.03, 2008).

6. 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

7. 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

8. 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)

9. 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

10. 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)

11. 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

12. 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.

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14. 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. *Journal of the Medical Association of Thailand.* **89** (9), 1528-1535

15. 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.

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18. Chuankhayan P, Hua Y, Svasti J, Sakdarat S, Sullivan PA, and Ketudat Cairns JR. 2005. Purification of an Isoflavonoid 7-O-β-apiosyl-glucoside β-glycosidase and its substrates from *Dalbergia nigrescens* Kurz. *Phytochemistry* **66,** 1880-1889. DOI:10.1016/j.phytochem.2005.06.024

19. 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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21. 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.

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24. 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.

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26. 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.

27. 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.

28. 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

)passiri, 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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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

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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)

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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

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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.

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

- 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

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

7.2 Current Research Projects:

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

8. Publications:

1. Seshadri S, Akiyama T, Opassiri R, Kuaprasert B, and Ketudat Cairns J. 2009. Structural and enzymatic characterization of Os3BGlu6, a rice β-glucosidase hydrolyzing hydrophobic glycosides and (1→3)- and (1→2)-linked disaccharides *Plant Physiology*; **151,** 47-58. doi:10.1104/pp.109.139436 (ISI IF 6.110, 2008).

2. Akiyama T, Jin S, Yoshida M, Hoshino T, Opassiri R, Ketudat Cairns JR. 2009. Expression of an endo-(1,3;1,4)-beta-glucanase in response to wounding, methyl jasmonate, abscisic acid and ethephon in rice seedlings. *Journal of Plant Physiology* 2009 Jun 29. [Epub ahead of print] (ISI IF 2.437, 2008)

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5. 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.

6. 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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