รายงานวิจัยฉบับสมบูรณ์

โครงการความสัมพันธ์ระหว่างโครงสร้างและการทำงาน ของเอนไซม์เบต้า-กลูโคซิเดส จากพืชไทย

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Abstract

The project aims to study the structure-function relationship in the family of enzyme β-glucosidases using three Thai plants β-glucosidases as model enzymes. Dalcochinase is an isoflavonoid β-glucosidase, purified from Thai rosewood (*Dalbergia cochinchinensis* Pierre). Dnbglu2 is an isoflavonoid β-diglycosidase from blackwood (*D. nigrescens* Kurz). Linamarase is a cyanogenic β-glucosidase present in cassava (*Manihot esculenta* Crantz). These three enzymes catalyse the same hydrolytic reactions, but are remarkably different in their specificities for natural substrates. Dalcochinase can perform reverse hydrolysis well, and catalyse transglucosylation using primary and secondary alcohols as acceptor, but not tertiary alcohol. In contrast, linamarase is exceptional at catalyzing transglucosylation using primary, secondary and tertiary alcohols as acceptor, but poor in catalyzing reverse hydrolysis. Understanding the mechanisms underlying these differences should lead us to better applications of the enzymes via protein engineering.

In this project, recombinant dalcochinase and Dnbglu2 have been cloned, expressed in yeast *Pichia pastoris* and purified from culture medium. Both purified recombinant enzymes exhibited similar kinetic properties to their respective natural enzymes isolated from seeds. Site-directed mutagenesis was performed to investigate the roles of amino acid residues in the aglycone binding pocket of these enzymes that might be responsible for differences in their catalytic properties. Ten dalcochinase mutants, namely I185A, N189F, M195V, H253F, V255F, N323Q, G367S, K402Y, A454N and E455I, were made by replacing residues in dalcochinase with the corresponding residues in linamarase, and were expressed and purified from *P. pastoris* cultures. Kinetic studies showed that most mutant enzymes decreased their specificities toward *p*-nitrophenyl-β-D-glucopyranoside (a commercial substrate) and dalcochinin β-D-glucoside (a natural substrate of dalcochinase). Notably, a 70- and 22-fold reduction shown by I185A, toward each respective substrate, suggested the importance of residue I185 in dalcochinase for substrate specificity. However, the hydrolytic activities towards linamarin (a natural substrate of linamarase) did not improve. In transglucosylation studies, three mutants showed improved transglucosylation abilities: I185A and V255F with both primary and secondary alcohol acceptors, N189F with only primary alcohol acceptors. So, residues A201, F205 and F271 in linamarase seemed to play prominent roles in catalysing transglucosylation. However, none of the mutant enzyme could catalyze the transfer of glucose to tertiary alcohol. Thus, it is expected that mutations at more than one position may generate new dalcochinase mutants which may function similarly to linamarase, in terms of substrate specificity and transglucosylation.

Furthermore, the differences in the aglycone binding pocket of dalcochinase and Dnbglu2 are compared, since they exhibit marked differences in substrate specificities, despite having 81% sequence identity. Three dalcochinase mutants, namely A454S and E455G single mutants and A454S/E455G double mutant, were

made by replacing residues in dalcochinase with the corresponding residues in Dnbglu2, and were expressed and purified from *P. pastoris* cultures. Kinetic studies showed that the hydrolytic efficiency toward *p*-nitrophenyl-β-D-glucopyranoside of both single mutants were similar to that of dalcochinase, but increased 2-fold by the double mutant. Furthermore, their activities for dalcochinin β-glucoside remained unchanged. For hydrolysis of isoflavonoid diglycosides dalpatein 7-O-β-D-apiosyl-1,6-β-D-glucoside and dalnigrein 7-O-β-D-apiosyl-1,6-β-D-glucoside (natural substrates of Dnbglu2), the single mutants showed similar hydrolysis of compared with the wild-type dalcochinase, whereas the double mutant showed 4-7 fold increases in relative activity toward both isoflavonoid diglycosides. However, this increased activity was only 3% that of Dnbglu2, indicating other determinants are important for isoflavonoid diglycoside hydrolysis.

As for cloning of cassava linamarase, four full-length cDNA sequences were obtained from RT-PCR and cloned into pPICZαB for methanol-inducible expression in *P. pastoris*, similar to the cloning strategies used for dalcochinase. However, no β-glucosidase activities could be detected in *P. pastoris* culture media. So these four cDNA sequences were to be cloned into pYEX-BX as an N-terminal fusion product with the signal sequence of yeast *SUC2* invertase followed by a polyhistidine tag for expression in *Saccharomyces cerevisiae* under the control of yeast copper metallothionein (*CUP1*) promoter.

Keywords

β-glucosidase, substrate specificity, transglucosylation, *Dalbergia cochinchinensis*, protein engineering, site-directed mutagenesis

บทคัดย่อ

โครงการวิจัยนี้มีวัตถุประสงค์เพื่อศึกษาความสัมพันธ์ระหว่างโครงสร้างและหน้าที่ในเอนไซม์กลุ่มเบต้า-กลูโคซิเดส โดยใช้เอนไซม์เบต้า-กลูโคซิเดสจากพืชไทย 3 ชนิดเป็นตัวอย่างศึกษา เอนไซม์ดัลโคชิเนส (dalcochinase) เป็นไอโซฟลาโวนอยด์ เบต้า-กลูโคซิเดสเอนไซม์ ที่สกัดได้จากเมล็ดพะยูง (*Dalbergia cochinchinensis* Pierre หรือ Thai Rosewood) เอนไซม์ Dnbglu2 เป็นไอโซฟลาโวนอยด์ เบต้า-ไดไกลโคซิเดสเอนไซม์ ที่สกัดได้จากเมล็ดฉนวน (*D. nigrescens* Kurz) เอนไซม์ลินามาเรส (linamarase) เป็นไซยาโนจีนิก เบต้า-กลูโคซิเดสเอนไซม์ ที่ได้จากมันสำปะหลัง (*Manihot esculenta* Crantz หรือ cassava) เอนไซม์ทั้งสามชนิดนี้เร่งปฏิกิริยาการสลายพันธะไกลโคซิดิกได้เหมือนกัน แต่แตกต่างกันในเรื่องความจำเพาะต่อสับสเตรทธรรมชาติ เอนไซม์ดัลโคชิเนสสามารถเร่งปฏิกิริยาย้อนการสลาย (reverse hydrolysis) ได้ดีและเร่งปฏิกิริยาการย้ายหมู่กลูโคส (transglucosylation) โดยใช้แอลกอฮอล์ชนิดปฐมภูมิและทุติยภูมิเป็นตัวรับได้ แต่ใช้แอลกอฮอล์ชนิดตติยภูมิไม่ได้ ในทางตรงกันข้าม เอนไซม์ลินามาเรสสามารถเร่งปฏิกิริยาการย้ายหมู่กลูโคสโดยใช้แอลกอฮอล์ชนิดปฐมภูมิ ทุติยภูมิ และตติยภูมิเป็นตัวรับได้ดีมาก แต่เร่งปฏิกิริยาย้อนการสลายได้น้อย การเข้าใจถึงกลไกของความแตกต่างเหล่านี้ จะสามารถนำไปสู่การประยุกต์ใช้เอนไซม์ได้ โดยอาศัยเทคนิคทางวิศวกรรมโปรตีน

ในโครงการวิจัยนี้ รีคอมบิแนนต์ดัลโคชิเนสและ Dnbglu2 ได้ถูกโคลนและแสดงออกในยีสต์ *Pichia pastoris* และสกัดให้บริสุทธิ์จากอาหารเลี้ยงเชื้อ เมื่อทำให้บริสุทธิ์แล้ว รีคอมบิแนนต์เอนไซม์ทั้งสองชนิดมีคุณสมบัติทางจลนพลศาสตร์ที่คล้ายคลึงกับเอนไซม์ที่สกัดได้จากเมล็ด เอนไซมรีคอมบิแนนต์ดัลโคชิเนสได้ถูกทำการกลายพันธุ์ที่ตำแหน่งจำเพาะ (site-directed mutagenesis) เพื่อศึกษาบทบาทของกรดอะมิโนในช่องจับกับอะไกลโคน ที่อาจทำหน้าที่กำหนดความแตกต่างในการเร่งปฏิกิริยา เอนไซม์ดัลโคชิเนสกลายพันธุ์ 10 ชนิด (I185A, N189F, M195V, H253F, V255F, N323Q, G367S, K402Y, A454N และ E455I) ได้ถูกสร้างขึ้น โดยการแทนที่กรดอะมิโนในเอนไซม์ดัลโคชิเนส ด้วยกรดอะมิโนที่ตำแหน่งเดียวกันในเอนไซม์ลินามาเรส และทำการแสดงออกแล้วสกัดให้บริสุทธิ์จากการเพาะเลี้ยง *P. pastoris* การศึกษาทางจลนพลศาสตร์พบว่า เอนไซม์กลายพันธุ์ส่วนใหญ่มีความจำเพาะต่อ *p*-nitrophenyl-β-D-glucopyranoside (สับสเตรททางการค้า) และ dalcochinin β-D-glucoside (สับสเตรทธรรมชาติของเอนไซม์ดัลโคชิเนส) ลดลง โดยเฉพาะเอนไซม์กลายพันธุ์ I185A ที่มีความจำเพาะลดลง 70 และ 22 เท่า ตามลำดับ ซึ่งชี้ถึงความสำคัญของกรดอะมิโนตำแหน่ง I185 ในเอนไซม์ดัลโคชิเนสในการกำหนดความจำเพาะต่อสับสเตรท อย่างไรก็ตาม ความสามารถในการสลายลินามาริน (linamarin ซึ่งเป็นสับสเตรทธรรมชาติของเอนไซม์ลินามาเรส) ไม่มีความเปลี่ยนแปลง ในการศึกษาปฏิกิริยาการย้ายหมู่กลูโคสพบว่า เอนไซม์กลายพันธุ์ 3 ชนิด มีความสามารถในการเร่งปฏิกิริยาการย้ายหมู่กลูโคสได้ดีขึ้น กล่าวคือ เอนไซม์กลายพันธุ์ I185A and V255F ใช้แอลกอฮอล์ชนิดปฐมภูมิและทุติยภูมิได้ดีขึ้น และเอนไซม์กลายพันธุ์ N189F ใช้แอลกอฮอล์ชนิดปฐมภูมิได้ดีขึ้น ดังนั้น กรดอะมิโนตำแหน่ง A201, F205 และ F271 ในเอนไซม์ลินามาเรส น่าจะมีบทบาทเด่นในการเร่งปฏิกิริยาการย้ายหมู่กลูโคส อย่างไรก็ตาม ไม่พบเอนไซม์ดัลโคชิเนสกลายพันธุ์ชนิดใดที่สามารถย้ายหมู่กลูโคสไปสู่แอลกอฮอล์ชนิดตติยภูมิ ดังนั้น จึงคาดว่าหากทำการกลายพันธุ์มากกว่าหนึ่งตำแหน่ง อาจจะทำให้ได้เอนไซม์ดัลโคชิเนสกลายพันธุ์ชนิดใหม่ ที่ทำงานได้คล้ายคลึงกับเอนไซม์ลินามาเรส ในด้านความจำเพาะต่อสับสเตรทและด้านการย้ายหมู่กลูโคส

นอกจากนี้ งานวิจัยได้เปรียบเทียบความแตกต่างในช่องจับกับอะไกลโคนของเอนไซม์ดัลโคชิเนส และเอนไซม์ Dnbglu2 เนื่องจากเอนไซม์ทั้ง 2 ชนิดมีความแตกต่างในด้านความจำเพาะต่อสับสเตรท ถึงแม้ว่าจะมีลำดับกรดอะมิโนคล้ายกันถึง

81% เอนไซม์ดัลโคชิเนสกลายพันธุ์ 3 ชนิด (กลายพันธุ์ตำแหน่งเดียว คือ A454 และ E455I และกลายพันธุ์สองตำแหน่ง คือ A454S/E455G) ได้ถูกสร้างขึ้น โดยการแทนที่กรดอะมิโนในเอนไซม์ดัลโคชิเนส ด้วยกรดอะมิโนที่ตำแหน่งเดียวกันในเอนไซม์ Dnbglu2 และทำการแสดงออกแล้วสกัดให้บริสุทธิ์จากการเพาะเลี้ยง *P. pastoris* การศึกษาทางจลนพลศาสตร์พบว่า เอนไซม์กลายพันธุ์ตำแหน่งเดียวมีประสิทธิภาพในการสลาย *p*-nitrophenyl-β-D-glucopyranoside คล้ายกับเอนไซม์ดัลโคชิเนสดั้งเดิม แต่เพิ่มขึ้น 2 เท่าในเอนไซม์กลายพันธุ์สองตำแหน่ง นอกจากนี้พบว่า กิจกรรมของเอนไซม์กลายพันธุ์ต่อ dalcochinin β-glucoside ไม่เปลี่ยนแปลง สำหรับการสลาย dalpatein 7-O-β-D-apiosyl-1,6-β-D-glucoside และ dalnigrein 7-O-β-D-apiosyl-1,6-β-D-glucoside (สับสเตรทธรรมชาติของเอนไซม์ Dnbglu2) พบว่าเอนไซม์กลายพันธุ์ตำแหน่งเดียวสามารถสลายไอโซฟลาโวนอยด์ เบต้า-ไดไกลโคไซด์ 2 ชนิดนี้ ได้ดีใกล้เคียงกับเอนไซม์ดัลโคชิเนสดั้งเดิม ในขณะที่เอนไซม์กลายพันธุ์สองตำแหน่งมีความสามารถเพิ่มขึ้น 4-7เท่า อย่างไรก็ตาม ความสามารถที่เพิ่มขึ้นนี้ คิดเป็นแค่ 3% ของเอนไซม์ Dnbglu2 ซึ่งแสดงว่ามีส่วนอื่นของเอนไซม์ที่สำคัญต่อการกำหนดความสามารถในการสลายไอโซฟลาโวนอยด์ ไดไกลโคไซด์

ในส่วนของการโคลนเอนไซม์ลินามาเรสจากมันสำปะหลัง ได้ทำการโคลน full-length cDNA sequences 4 โคลน จากการทำ RT-PCR และโคลนเข้าสู่เวคเตอร์ pPICZαB เพื่อให้เกิดการแสดงออกใน *P. pastoris* โดยใช้เมทานอลเป็นตัวเหนี่ยวนำ ซึ่งคล้ายกับวิธีการโคลนเอนไซม์ดัลโคชิเนส อย่างไรก็ตาม ไม่พบกิจกรรมของเอนไซม์เบต้า-กลูโคซิเดสในอาหารเลี้ยงเชื้อ *P. pastoris* ดังนั้น full-length cDNA sequences 4 โคลนนี้ จึงถูกโคลนเข้าในเวคเตอร์ pYEX-BX ในรูปของ N-terminal fusion product กับลำดับของ the signal sequence ของ yeast *SUC2* invertase ตามด้วย polyhistidine tag และควบคุมการแสดงออกโดย copper metallothionein (*CUP1*) promoter

คำสำคัญ
เบต้า-กลูโคซิเดส ความจำเพาะต่อสับสเตรท การย้ายหมู่กลูโคส พะยูง วิศวกรรมโปรตีน การกลายพันธุ์ที่ตำแหน่งจำเพาะ

Executive Summary

β-Glucosidases (E.C. 3.2.1.21) belong to a group of enzymes that catalyse the hydrolysis of β–O-glucosidic linkages between D-glucose and an aglycone or another sugar. All members of this enzyme family recognise a β-glucoside as their substrate. However, β-glucosidases from different sources differ vastly in their specificities for the aglycone linked to the glucosyl group. Furthermore, they are different in their abilities to catalyse reverse hydrolysis and glucose transfer reactions. It is likely that these differences occurred as a result of the specific interactions between unique amino acid residues in the active pocket and the specific substrates. Thus, the present research project employs techniques in molecular biology and protein engineering to study the structure-function relationship in the family of β-glucosidases. Understanding the mechanisms that determine these differences should lead us to better applications of the enzymes via development of engineered β-glucosidases with desired properties.

Three β-glucosidases from Thai plants are used as model enzymes in this study. Two β-glucosidases are from related *Dalbergia* species, namely dalcochinase from Thai rosewood (*Dalbergia cochinchinensis* Pierre), and Dnbglu2 from blackwood (*D. nigrescens* Kurz). Another β-glucosidase, linamarase, is a cyanogenic β-glucosidase present in cassava (*Manihot esculenta* Crantz). While all three enzymes catalyse the same hydrolytic reactions, they show remarkable differences in their specificity for substrate, and their catalytic activities in hydrolysis, reverse hydrolysis and transglucosylation reactions. Dalcochinase and Dnbglu2 show 81% sequence identity and yet their substrate specificities are distinct. Dalcochinase could hydrolyse dalcochinin β-D-glucoside, its natural substrate, efficiently, but not the two isoflavonoid diglycosides, the natural substrates of Dnbglu2, and vice versa. In transglucosylation, dalcochinase can transfer glucose from a glucosyl donor to primary and secondary alcohol acceptors, while linamarase exhibits a unique ability to catalyze glucose transfer to primary, secondary as well as tertiary alcohols as glucosyl acceptors. Previously, the coding sequence of dalcochinase has been cloned and expressed efficiently in yeast *Pichia pastoris*. Purified recombinant dalcochinase exhibits enzymatic properties that are similar to those of natural dalcochinase. The project now focuses on characterisation and site-directed mutagenesis of dalcochinase by replacing residues in dalcochinase with the corresponding residues in Dnbglu2 or linamarase. The effect of mutations will be tested to identify the residues that are important for differences in catalytic properties of dalcochinase and other β-glucosidases.

In the first part of this project, the sequence of wild-type Thai rosewood dalcochinase has been expressed and purified. The expression and purification procedures overcome the limitations in enzyme supply from natural sources, and allow further studies on structure-function relationships in this enzyme. To identify residues that are important for distinct properties of dalcochinase and cassava linamarase in substrate

specificities and transglucosylation activities, ten single mutations were made by site-directed mutagenesis to replace ten different residues in the aglycone binding pocket of dalcochinase with the corresponding residues of linamarase. This resulted in ten dalcochinase mutants, namely I185A, N189F, M195V, H253F, V255F, N323Q, G367S, K402Y, A454N and E455I. All mutants have been cloned into *P. pastoris*, expressed, purified, and characterized with respect to their substrate specificities and transglucosylation activities.

Kinetic studies showed showed variable effects of these ten single mutations on substrate specificity. Notably, the efficiency for hydrolysis of *p*-nitrophenyl-β-D-glucopyranoside (a commercial substrate) is lowered by 70-fold in I185A, but increased by 5- and 2-fold in N189F and A454N, respectively, when compared with the wild-type dalcochinase. Most dalcochinase mutants showed decreased in specificity for dalcochinin β-D-glucoside (a natural substrate of dalcochinase), especially a 22-fold reduction in I185A and a 12-fold reduction in V255F, when compared with the wild-type dalcochinase. Unexpectedly, E455I showed a 5-fold increase in specificity for dalcochinin β-D-glucoside, possibly indicating a preferable interaction between the rotenoid moiety of substrate and the hydrophobic side chain of isoleucine at this position. However, none of the dalcochinase mutants could hydrolyze linamarin (a natural substrate of linamarase), suggesting that the change in the overall conformation of the aglycone binding pocket, rather than site-specific changes, may be needed for the successful entry and hydrolysis of linamarin by dalcochinase mutants.

In transglucosylation studies, three mutants showed increased transglucosylation activities, namely I185A with long-chain primary and secondary alcohols, N189F with all primary alcohols, and V255F with both primary and secondary alcohols. However, none of the dalcochinase mutants could transfer glucose to tertiary alcohols, the property that is unique to cassava linamarase. Again, the change in the overall conformation of the aglycone binding pocket, rather than site-specific changes, may be needed for the successful entry and transglucosylation of tertiary alcohols by dalcochinase mutants. Thus, it is expected that mutations at more than one position may generate new dalcochinase mutants, which may function similarly to linamarase, in terms of substrate specificity and transglucosylation.

The second part of this project has been done in collaboration with Associate Professor James R. Ketudat Cairns at Schools of Biochemistry and Chemistry, Suranaree University of Technology. In this part, a cDNA encoding Dnbglu2 was also cloned and expressed in *P. pastoris*. Purified recombinant Dnbglu2 showed similar enzymatic properties to those of natural enzyme. The recombinant Dnbglu2 was less efficient at hydrolysis of both dalpatein 7-O-β-D-apiosyl-1,6-β-D-glucoside and dalnigrein 7-O-β-D-apiosyl-1,6-β-D-glucoside (natural substrates of Dnbglu2) compared to natural Dnbglu2 from seed. Nonetheless, recombinant Dnbglu2 maintains a high preference for both isoflavonoid diglycosides over dalcochinin β-D-glucoside (a natural substrate of dalcochinase).

Also, in this part of the project, three more dalcochinase mutants, namely A454S and E455G single mutants and A454S/E455G double mutant, were made by replacing residues in the aglycone binding pocket of dalcochinase with residues of blackwood Dnbglu2 to identify residues that are important for distinct substrate specificities between these two enzymes. All mutants have been cloned into *P. pastoris*, expressed, purified, and characterized with respect to their substrate specificities. The single mutants of dalcochinase showed similar hydrolysis of dalpatein 7-O-β-D-apiosyl-1,6-β-D-glucoside and half the rate of hydrolysis of dalnigrein 7-O-β-D-apiosyl-1,6-β-D-glucoside compared with the wild-type dalcochinase. The double mutant showed 4- and 6.8-fold increases in relative activity toward dalpatein 7-O-β-D-apiosyl-1,6-β-D-glucoside and dalnigrein 7-O-β-D-apiosyl-1,6-β-D-glucoside, respectively. However, this activity was only 3% that of Dnbglu2, indicating other determinants are important for isoflavonoid diglycoside hydrolysis. The results from this part of the project have been reported in Archives of Biochemistry and Biophysics, 468 (2007) 205–216 (Appendix 1).

In the third part of this project, the cDNA of cassava linamarase was cloned and sequenced for future site-directed mutagenesis and characterization. Six different cDNA sequences of linamarase from cassava were amplified by reverse transcription-polymerase chain reaction (RT-PCR) using primers designed from the previously reported cDNA sequence of cassava linmarase. Deduced amino acid sequences from four cDNA clones showed 98-99% sequence identity to that of pCAS5, while the other two cDNA clones contained nucleotide sequences that led to premature termination. The four full-length cDNA sequences were inserted into pPICZαB for methanol-inducible expression in *P. pastoris* as a secretory protein with the alpha mating factor propeptide and the polyhistidine tag at the N-terminus, similar to the cloning strategies used for dalcochinase. However, none of them showed significant β-glucosidase activities in *P. pastoris* culture media. A new cloning strategy has been devised to clone the four cDNA sequences in pYEX-BX as an N-terminal fusion product with the signal sequence of yeast *SUC2* invertase followed by a polyhistidine tag, and to express the recombinant protein in *Saccharomyces cerevisiae* under the control of yeast copper metallothionein (*CUP1*) promoter. The results from this part of the project have been reported in Kasetsart Journal (Natural Science), 40 (2006) 123-128 (Appendix 2).

เนื้อหางานวิจัย

1. Production of recombinant Thai rosewood β-glucosidase (dalcochinase)

The coding sequence of an N-terminally truncated form of recombinant dalcochinase was cloned with a polyhistidine-tag after the N-terminal alpha factor signal sequence, and expressed in *P. pastoris* under an inducible *AOX1* promoter. The recombinant enzyme could be efficiently purified via hydrophobic interaction chromatography and immobilized metal-ion affinity chromatography (IMAC) (Fig. 1). The enzymatic properties of recombinant dalcochinase were similar to those of natural dalcochinase. The production of recombinant dalcochinase enables further investigations into the structure-function relationships in this enzyme via site-directed mutagenesis. The procedures for cloning, expression, purification and enzymatic characterization have been reported in Toonkool et al. (2006). This preparation of recombinant dalcochinase will be used to test properties of native enzyme, compared with mutant enzymes.

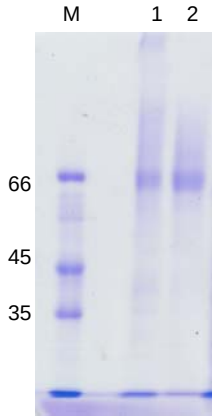

Figure 1: SDS-PAGE of purified recombinant dalcochinase. Lane M, protein standards (kD); lanes 1-2, recombinant wild-type dalcochinase.

2. Identification of amino acid residues for site-directed mutagenesis

Due to the lack of three-dimensional structures of the three β-glucosidases in this study, their three-dimensional models were generated using Geno3D (geno3d-pbil.ibcp.fr). The crystal structure of maize β-glucosidase ZmGlu1 (PDB code 1E56A), which showed ~40% sequence identity to these three enzymes, was used as a template. The overall structures of the models were checked by Ramachandran plot and z-score using PROCHECK and ProSA, respectively. The Ramachandran plot showed that 76.2% of amino acid residues of dalcochinase were located in the most favorable region and only 1.5% in the disallowed region. Generally, models in which less than 2% of amino acid residues were located in the disallowed region are considered as reliable and were chosen for further study. The z-score of selected dalcochinase model was

also within the acceptable range. The three-dimensional models of dalcochinase (Fig. 2), Dnbglu2 and linamarase (not shown) exhibited similar $(\beta/\alpha)_8$ barrel structures as expected for enzymes in glycosyl hydrolase family 1.

Figure 2: The three-dimensional model of dalcochinase. The catalytic acid/base residues (E182) and nucleophile residues (E396) of dalcochinase are shown as stick drawings with labels.

To identify the target residues in dalcochinase for site-directed mutagenesis, comparison has been made between amino acid sequences of dalcochinase, Dnbglu2 and linamarase using ClustalW 2.0.8 (Fig. 3). Dalcochinase showed 81% and 47% sequence identity to Dnbglu2 and linamarase, respectively. The highly conserved (L/F)NEP and (I/V)TENG motifs, with two glutamate residues acting as the acid/base catalyst and nucleophile in the hydrolysis, respectively, form parts of the glycone binding pocket.

As a result of sequence alignment and structural modeling, amino acid residues were chosen for site-directed mutagenesis based on 2 criteria: a) their location in the aglycone binding pocket, and (b) being different in the two enzymes. To study the differences in substrate specificity and transglucosylation between dalcochinase and linamarase, ten amino acid residues in dalcochinase, namely I185, N189, M195, H253, V255, N323, G367, K402, A454 and E455, were identified as targets for site-directed mutagenesis, and would be replaced with the residues of linamarase at the corresponding positions. This would generate ten single mutants of dalcochinase, which are I185A, N189F, M195V, H253F, V255F, N323Q, G367S, K402Y, A454N and E455I.

To study the differences in substrate specificity between dalcochinase and Dnbglu2, two amino acid residues in dalcochinase, namely A454 and E455, were identified as targets for site-directed mutagenesis, and would be replaced with the residues of Dnbglu2 at the corresponding positions. This would generate 2 single mutants (A454S and E455G) and one double mutant (A454S/E455G) of dalcochinase. This part of the project

has been done in collaboration with Associate Professor James R. Ketudat Cairns at Schools of Biochemistry and Chemistry, Suranaree University of Technology.

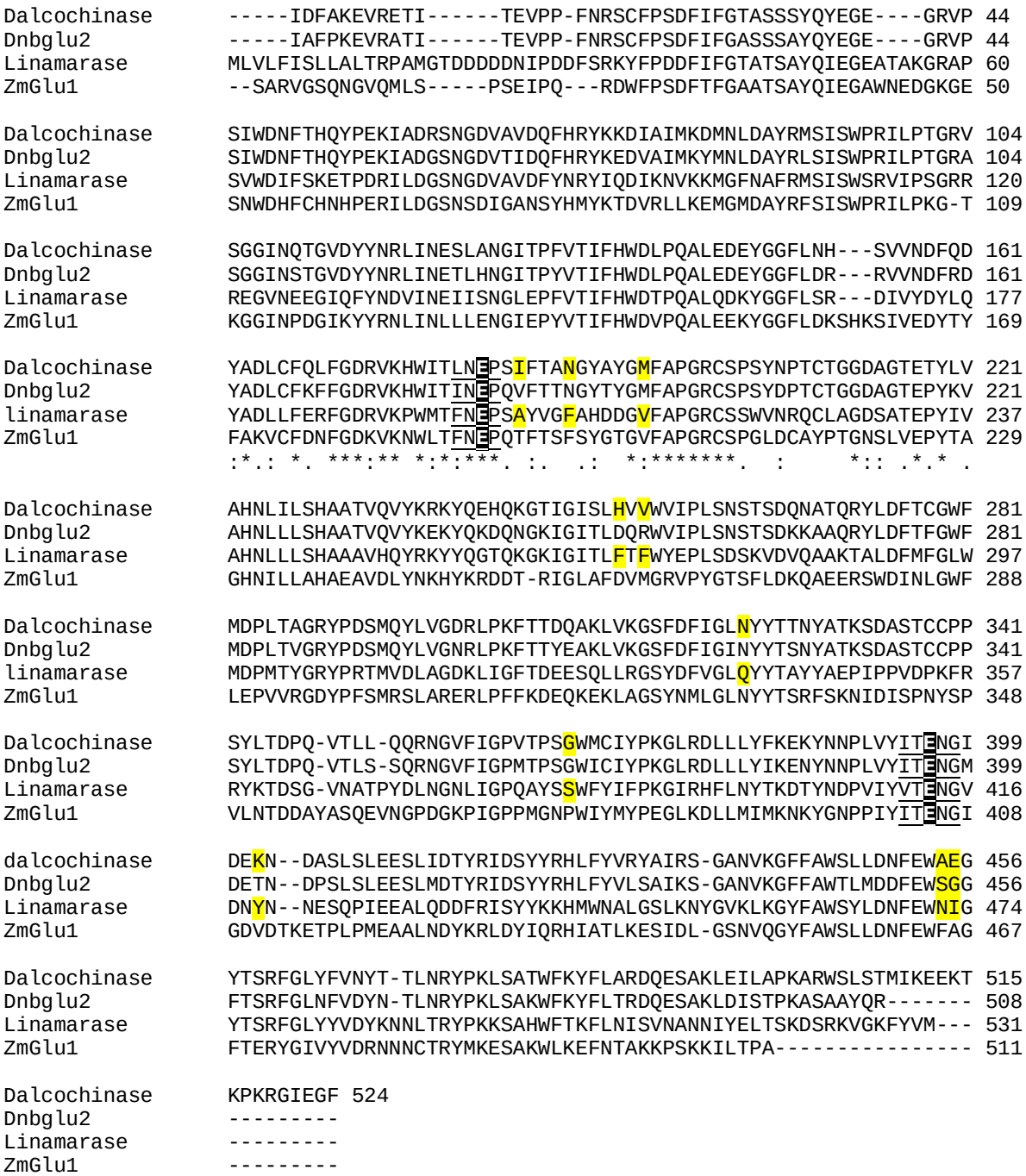

Figure 3: Alignment of amino acid sequences of dalcochinase, Dnbglu2, linamarase and ZmGlu1 generated by ClustalW 2.0.8. The highly conserved (L/F)NEP and (I/V)TENG motifs are underlined, and the two catalytic glutamate residues are shown as white letters against black background. The residues that are targeted for site-directed mutagenesis are shaded.

3. Site-directed mutagenesis and production of mutant enzymes

Site-directed mutagenesis has been performed to make mutant forms of dalcochinase. All mutant constructs have been confirmed by DNA sequencing. The mutant constructs were transformed into *P. pastoris*, expressed, and purified to homogeneity according to the procedures used for wild-type recombinant dalcochinase (Toonkool et al., 2006). They were subjected to SDS-PAGE, western blot analyses and activity-stained non-denaturing PAGE analyses. Fig. 4 shows SDS-PAGE and western blot analysis of some of the purified dalcochinase mutants compared with the natural and recombinant wild-type dalcochinase. As expected, all dalcochinase mutants gave a broad band with an apparent molecular weight of approximately 66 kD on SDS-PAGE (Fig. 4, A, lanes 3-4), similar to recombinant wild-type dalcochinase (Fig. 4, A, lane 2). All mutants showed immunoreactivity against mouse monoclonal antibody against natural dalcochinase (Fig. 4, B, lanes 3-4), as seen with the natural and recombinant dalcochinase (Fig. 4, B, lanes 1-2). Mutant forms of dalcochinase showed variable activity when their non-denaturing PAGE was stained with 1 mM 4-MU-Glc (results not shown).

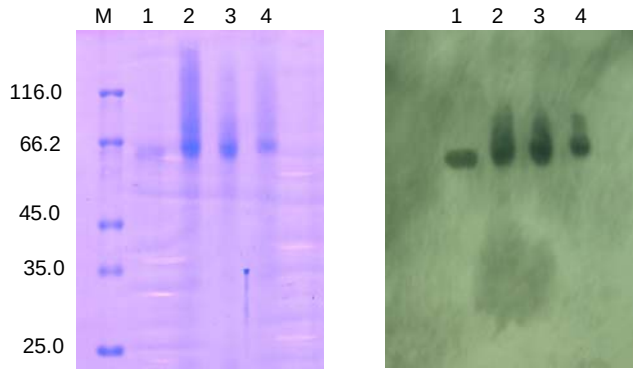

Figure 4: Example of SDS-PAGE of some purified enzymes. (A) coomassie stain and (B) western blot. Lane M, protein standards (kD); lane 1, natural dalcochinase; lane 2, recombinant wild-type dalcochinase; lane 3, A454N; lane 4, N189F.

4. Kinetic study of dalcochinase mutants

4.1 Dalcochinase versus linamarase

To study the differences in substrate specificity between dalcochinase and linamarase, the hydrolytic activities of ten dalcochinase mutants, each containing a residue of linamarase in its aglycone binding pocket, were characterised, and compared with natural and recombinant wild-type enzymes. Kinetic parameters of these enzymes toward *p*-nitrophenyl-β-D-glucopyranoside (*p*NP-Glc, a commercial substrate) and dalcochinin β-D-glucoside (a natural substrate of dalcochinase) are shown in Table 1.

The kinetic studies showed variable effects of mutations in the aglycone binding pocket of dalcochinase on enzyme specificity toward *p*NP-Glc and dalcochinin β-D-glucoside substrates (Table 1). Natural and recombinant wild-type dalcochinase showed similar kinetic properties as expected. For hydrolysis of *p*NP-Glc, natural linamarase was 3 times more efficient than the wild-type dalcochinase (natural and recombinant enzymes), with a 5.4-fold lowered K_m and a 1.7-fold lowered k_{cat}. Most mutants were less efficient in hydrolysis of *p*NP-Glc, especially a 70- and 34-fold reduction by I185A and G367S, respectively, compared with the recombinant wild-type dalcochinase. However, the efficiency for hydrolysis of *p*NP-Glc was increased by 5- and 2-fold in N189F and A454N, respectively, compared with the recombinant wild-type dalcochinase. While the effects on the affinity to *p*NP-Glc were rather variable and mild (less than an order of magnitude), the turn-over numbers of all dalcochinase mutants were lowered than the wild-type dalcochinase, following the same trend as natural linamarase. In particular, I185A and G367S showed 18- and 24-fold reduction in k_{cat} compared with the recombinant wild-type dalcochinase, while N189F and A454N gave similar k_{cat} values to linamarase. These effects indicate a change in steric and/or polar complementarities between the aglycone binding pocket of enzymes and the *p*NP moiety of substrate.

Table 1: Kinetic parameters of natural and recombinant dalcochinase, natural linamarase, and dalcochinase mutants toward *p*NP-Glc and dalcochinin β-D-glucoside.

Substrate	*p*NP-Glc			Dalcochinin β-D-glucoside		
Enzymes	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($s^{-1}M^{-1}$)	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($s^{-1}M^{-1}$)
Natural dalcochinase	3.92	274.43	70,000	6.78	90.63	13,000
Recombinant dalcochinase	3.91	225.49	58,000	5.67	62.25	11,000
Natural linamarase	0.72	131.91	183,000	-	-	-
I185A	14.33	12.29	860	1.62	0.74	500
N189F	0.59	172.51	292,000	0.63	4.00	6,300
M195V	1.77	36.38	20,500	6.25	31.44	5,000
H253F	2.69	58.01	21,600	4.35	21.92	5,000
V255F	2.67	10.60	4,000	1.74	1.55	900
N323Q	1.84	36.21	20,000	4.94	33.52	6,800
G367S	5.53	9.34	1,700	1.62	1.73	1,100
K402Y	2.61	15.49	6,000	3.60	4.00	1,100
A454N	1.34	157.90	118,000	4.69	9.19	2,000
E455I	11.83	49.85	4,200	3.63	192.39	53,000

The replacement of an amino acid residue of linamarase in the aglycone binding pocket of dalcochinase also showed variable effects on the specificity toward dalcochinin β-D-glucoside. Most mutants showed between 2-22 fold reduction in its specificity compared with the recombinant wild-type dalcochinase, following the same trend as natural linamarase, which could not appreciably hydrolyse dalcochinin β-D-glucoside. Most notable reduction was by I185A, V255F, G367S and K402Y (22-, 12-, 10- and 10-fold, respectively), suggesting close interactions between residues in the aglycone binding pockets of dalcochinase and the rotenoid moiety of substrate. Unexpectedly, E455I showed a 5-fold increase in specificity for dalcochinin β-D-glucoside, possibly indicating a preferable interaction between the rotenoid moiety of substrate and the hydrophobic side chain of isoleucine at this position. Again, the effects on the turn-over numbers were more pronounced than the effects on the affinity of enzymes for dalcochinin β-D-glucoside, which surprisingly was not significantly affected by the mutations (less than an order of magnitude). So interactions between residues in the aglycone binding pockets of dalcochinase and the rotenoid moiety of substrate seemed to be flexible in terms of binding, but contribute significantly in terms of catalysis.

For hydrolysis of linamarin (a natural substrate of linamarase), natural linamarase exhibited a K_m 2.28 mM, k_{cat} of 47.03 s^{-1}, and k_{cat}/K_m of 20,600 $s^{-1}M^{-1}$. However, none of the wild-type or mutant constructs of dalcochinase could hydrolyze linamarin, suggesting that the change in the overall conformation of the aglycone binding pocket, rather than site-specific changes, may be needed for the successful entry and hydrolysis of linamarin by dalcochinase mutants. Thus, it is expected that mutations at more than one position may generate new dalcochinase mutants with similar substrate specificity to linamarase.

4.2 Dalcochinase versus Dnbglu2

To study the differences in substrate specificity between dalcochinase and Dnbglu2, two residues in the aglycone binding pocket of dalcochinase were replaced with the corresponding residue of Dnbglu2, generating 2 single mutants (A454S and E455G) and one double mutant (A454S/E455G) of dalcochinase. The hydrolytic activities of dalcochinase mutants toward *p*NP-Glc, dalcochinin β-D-glucoside, and dalpatein 7-O-β-D-apiosyl-1,6-β-D-glucoside and dalnigrein 7-O-β-D-apiosyl-1,6-β-D-glucoside (natural substrates of Dnbglu2), were characterised, and compared with natural and recombinant wild-type enzymes. This part of the project has been done in collaboration with Associate Professor James R. Ketudat Cairns at Schools of Biochemistry and Chemistry, Suranaree University of Technology.

The single mutants of dalcochinase showed similar hydrolysis of dalpatein 7-O-β-D-apiosyl-1,6-β-D-glucoside and half the rate of hydrolysis of dalnigrein 7-O-β-D-apiosyl-1,6-β-D-glucoside compared with the wild-type dalcochinase. The double mutant showed 4- and 6.8-fold increases in relative activity toward dalpatein 7-O-β-D-apiosyl-1,6-β-D-glucoside and dalnigrein 7-O-β-D-apiosyl-1,6-β-D-glucoside, respectively. However, this

activity was only 3% that of Dnbglu2, indicating other determinants are important for isoflavonoid diglycoside hydrolysis.

5. Transglucosylation study of dalcochinase mutants

To study the differences in transglucosylation specificity between dalcochinase and linamarase, the transglucosylation activities of ten dalcochinase mutants, each containing a residue of linamarase in its aglycone binding pocket, were compared with natural and recombinant wild-type enzymes. The transglucosylation reaction used 10 mM *p*NP-Glc as a glucosyl donor and 0.9 M short chain alkyl alcohols as a glucosyl acceptor. The % mole of alkyl glucoside products are shown in Table 2.

Table 2: Percent mole of alkyl glucoside products from transglucosylation reactions.

Alcohol	Primary					Secondary		Tertiary
Enzymes	Methanol	Ethanol	*n*-Propanol	*n*-Butanol	*iso*-Butanol	*iso*-Propanol	*sec*-Butanol	*tert*-Butanol
Natural dalcochinase	24	38	66	83	69	4	12	-
Recombinant dalcochinase	22	42	68	79	71	4	12	-
Natural linamarase	52	51	73	71	74	60	65	35
I185A	20	40	73	92	90	18	25	-
N189F	43	64	86	96	90	7	11	-
M195V	24	46	68	90	71	3	10	-
H253F	29	44	66	87	75	6	11	-
V255F	40	54	80	92	84	15	21	-
N323Q	29	43	69	88	74	6	12	-
G367S	30	36	58	73	62	9	8	-
K402Y	25	40	65	89	74	6	13	-
A454N	29	50	68	85	75	4	15	-
E455I	26	40	67	87	80	7	10	-

In transglucosylation studies, natural and recombinant wild-type dalcochinase showed similar transglucosylation activities as expected. Natural linamarase was much better than any enzymes in transglucosylation to all primary, secondary and tertiary alcohols acceptors. Most mutants gave similar levels of % mole of alkyl glucosides from the reactions to the recombinant wild-type dalcochinase. Three mutants showed increased transglucosylation activities, namely I185A with long-chain primary and secondary alcohols, N189F with all primary alcohols, and V255F with both primary and secondary alcohols. Notably,

these three mutants gave higher yields of alkyl glucosides made from long-chain primary alcohols than natural linamrase, but those made from secondary alcohols were still lower. However, none of the dalcochinase mutants could transfer glucose to tertiary alcohols, the property that is unique to cassava linamarase. Again, the change in the overall conformation of the aglycone binding pocket, rather than site-specific changes, may be needed for the successful entry and transglucosylation of tertiary alcohols by dalcochinase mutants. Thus, it is expected that mutations at more than one position may generate new dalcochinase mutants with similar to transglucosylation activity to linamarase.

6. Cloning and expression of recombinant Dnbglu2

This part of the project has been done in collaboration with Associate Professor James R. Ketudat Cairns at Schools of Biochemistry and Chemistry, Suranaree University of Technology. In this part, a cDNA encoding Dnbglu2 was also cloned and expressed in *P. pastoris*. Purified recombinant Dnbglu2 showed similar enzymatic properties to those of natural enzyme. The recombinant Dnbglu2 was less efficient at hydrolysis of both dalpatein 7-O-β-D-apiosyl-1,6-β-D-glucoside and dalnigrein 7-O-β-D-apiosyl-1,6-β-D-glucoside compared to natural Dnbglu2 from seed. Nonetheless, recombinant Dnbglu2 maintains a high preference for both isoflavonoid diglycosides over dalcochinin β-D-glucoside (a natural substrate of dalcochinase).

7. Cloning and expression of recombinant cassava linamarase

Many isozymes of cassava linamarase exist, but only one cDNA sequence (pCAS5) has been reported thus far. In order to study the structure-function relationships in this enzyme, the cDNA of cassava linamarase was cloned and sequenced. Six different cDNA sequences of linamarase from cassava were amplified by reverse transcription-polymerase chain reaction (RT-PCR) using primers designed from the sequence of pCAS5, which is the previously reported cDNA sequence of cassava linmarase by Hughes et al. (1992). Deduced amino acid sequences from four cDNA clones showed 98-99% sequence identity to that of pCAS5, while the other two cDNA clones contained nucleotide sequences that led to premature termination. The four full-length cDNA sequences were inserted into pPICZαB for methanol-inducible expression in *P. pastoris* as a secretory protein with the alpha mating factor propeptide and the polyhistidine tag at the N-terminus, similar to the cloning strategies used for dalcochinase. However, none of them showed significant linamarase activities in *P. pastoris* culture media. A new cloning strategy has been devised to clone the four cDNA sequences in pYEX-BX as an N-terminal fusion product with the signal sequence of yeast *SUC2* invertase followed by a polyhistidine tag, and to express the recombinant protein in *Saccharomyces cerevisiae* under the control of yeast copper metallothionein (*CUP1*) promoter.

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Kongsaeree, P.T., Ratananikom, K., Choengpanya, K., Sujiwattanarat, P., Porncharoennop, C., Onpium, A., Saparpakorn, P. and Svasti, J. Substrate specificity for hydrolysis and transglucosylation in glycosyl hydrolase family 1. (*in preparation*)

Publication in national journal

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ภาคผนวก 1

Chuankhayan, P., Rimlumduan, T., Tantanuch, W., Mothong, N., Kongsaeree, P.T., Metheenukul, P., Svasti, J., Jensen, O.N. and Ketudat Cairns, J.R. Functional and structural differences between isoflavonoid β-glycosidases from *Dalbergia sp*. Archives of Biochemistry and Biophysics 2007; 468: 205–216.

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Functional and structural differences between isoflavonoid β-glycosidases from *Dalbergia* sp.

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Abstract

Among isoflavonoid β-glucosidases from *Dalbergia* species, that from *Dalbergia nigrescens* hydrolyzes isoflavonoid-7-*O*-β-D-apiosyl-1,6-β-D-glucosides more efficiently, while *Dalbergia cochinchinensis* β-glucosidase (dalcochinase) hydrolyzes its rotenoid glycoside substrate, dalcochinin β-D-glucoside (I), more efficiently. A cDNA encoding a glycosylated β-glucosidase with 81% identity with dalcochinase was cloned from *D. nigrescens* seeds, and its protein (Dnbglu2) expressed in *Pichia pastoris*. Purified Dnbglu2 hydrolyzed the *D. nigrescens* natural substrates dalpatein 7-*O*-β-D-apiofuranosyl-(1 → 6)-β-D-glucopyranoside (II) and dalnigrein 7-*O*-β-D-apiofuranosyl-(1 → 6)-β-D-glucopyranoside (III) at 400- and 5000-fold higher catalytic efficiency (k_{cat}/K_m) than I. Dalcochinase was mutated at two amino acid residues, A454S and E455G, that are homologous to previously described substrate binding residues and differ from the corresponding residues in Dnbglu2. The double mutant showed 4- and 6.8-fold increases in relative activity toward II and III, respectively. However, this activity was only 3% that of Dnbglu2 β-glucosidase, indicating other determinants are important for isoflavonoid diglycoside hydrolysis.

Keywords: *Dalbergia nigrescens*; *Dalbergia cochinchinensis*; β-Glucosidase; Isoflavonoid glycoside; Diglycosidase; Glycosyl hydrolase family 1; Substrate specificity; Glycosylation; Plant; 7-*O*-β-D-Apiofuranosyl-(1 → 6)-β-D-glucopyranoside

Glycosyl hydrolase family 1 (GH1)[2] includes enzymes with a variety of glycone (sugar) specificities, including β-glucosidases (EC 3.2.1.21), β-fucosidases (EC 3.2.1.38), β-galactosidases (EC 3.2.1.23), β-mannosidases (EC 3.2.1.25), phospho-β-glucosidases (EC 3.2.1.86), and phospho-β-galactosidases (EC 3.2.1.85) [1]. Some of these enzymes show very narrow sugar specificity, while others hydrolyze a range of sugars from glycosides. Recently, diglycosidases have been added to this list [2].

Monoglycosidases, such as β-glucosidases and β-galactosidases, have been widely reported in plants and microorganisms, but not many diglycosidases have been reported in higher plants. Diglycosidases that have been studied include *Camellia sinensis* β-primeverosidase (EC 3.2.1.149) [2,3], *Rhamnus dahurica* rhamnodiastase [4],

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[2] *Abbreviations used:* GH1, glycosyl hydrolase family 1; PGO, glucose-oxidase/peroxidase mixture; PVPP, 2-mercaptoethanol, polyvinyl-polypyrolidone; PMSF, phenylmethylsulfonyl fluoride; TdT, terminal deoxynucleotidyl transferase; RACE, rapid amplification of cDNA ends; NCBI, National Center for Biotechnology Information; *p*NP-Fuc, *p*-nitrophenyl-β-D-fucopyranoside; *p*NP, *p*-nitrophenol; IMAC, immobilized metal affinity chromatography.

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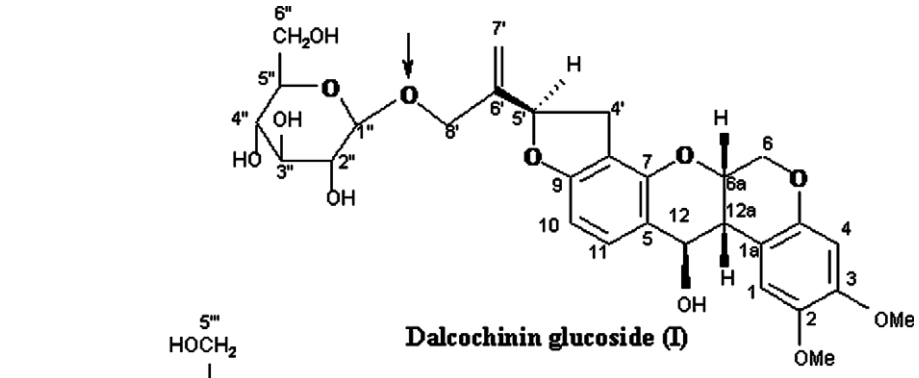
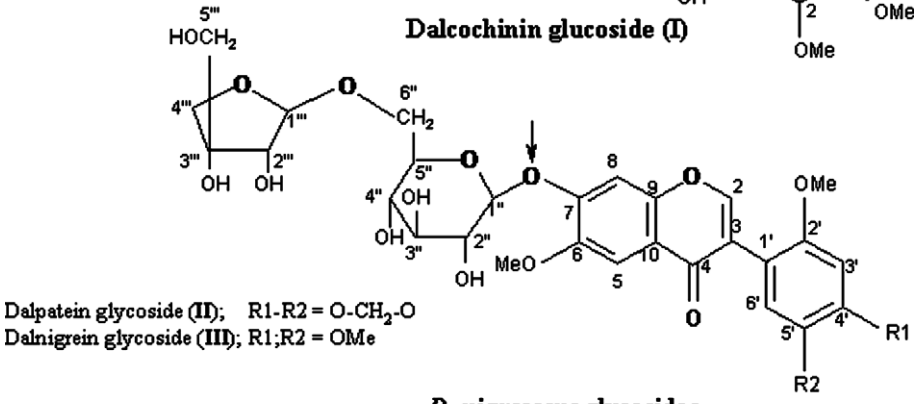
Viburnum furcatum furcatin hydrolase (EC 3.2.1.161) [5,6], *Fagopyrum esculentum* L. rutinase [7], *Fagopyrum tataricum* L. rutinase [8], and *Vicia angustifolia* vicianin hydrolase [9] in plants, and *Aspergillus flavas* (rutinase) in fungus [10].

Diglycosidases or disaccharide glycosidases directly hydrolyze the second β-glycosidic bond from the nonreducing end of disaccharide glycosides to liberate a disaccharide unit and an aglycone. For example, furcatin hydrolase hydrolyzes furcatin (*p*-allylphenyl 6-*O*-β-D-apiofuranosyl-β-D-glucopyranoside, Fig. 1(IV)) into *p*-allylphenol and the disaccharide acuminose (1,6-β-apiofuranosyl-glucopyranose, [6]) and β-primeverosidase hydrolyzes β-primeverosides to release primeverose (6-*O*-β-D-xylopyranosyl-β-D-glucopyranose) and alcoholic floral aroma derivatives [2,3]. Furcatin hydrolase shows low activity toward mono-

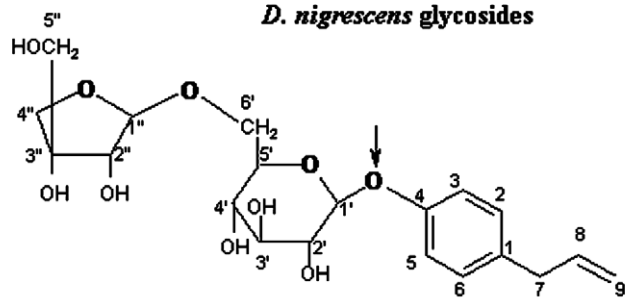


Dalcochinin glucoside (I)

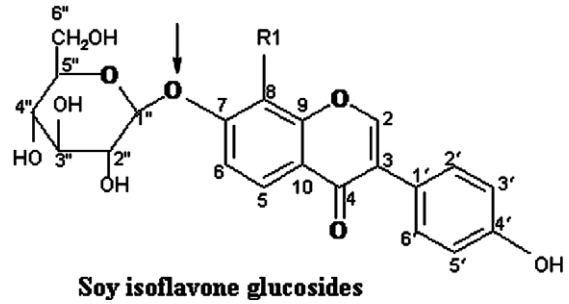


Dalpatein glycoside (II); R1-R2 = O-CH$_2$-O
Dalnigrein glycoside (III); R1;R2 = OMe

***D. nigrescens* glycosides**



Furcatin (IV)



Genistin; R1= OH (V)
Daidzin; R1= H (VI)

Soy isoflavone glucosides

Fig. 1. Structures of natural substrates of *Dalbergia* β-glycosidase to show the different positions of the bonds that are hydrolyzed. The position of the bond that is hydrolyzed is indicated by the arrow for each substrate. The *Dalbergia* glycosides shown are (I) dalcochinin β-D-glucoside, (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. For comparison, the structures of the disglycoside furcatin (VI) and the soy isoflavone glucosides genistin (V) and daidzin (VI) are included.

glycoside substrates [6]. It hydrolyzes *p*-allylphenol β-D-glucopyranoside at 43% of its rate toward furcatin, even though these substrates bear the same aglycone moieties. It hydrolyzes *p*NP-β-D-glucopyranoside (*p*NP-Glu) and *p*NP-β-gentiobioside at 3% and 4% of its furcatin hydrolysis activity, respectively, and does not hydrolyze other monosaccharide glycosides, such as *p*NP-β-D-xylopyranoside (*p*NP-Xyl), *p*NP-α-L-arabinopyranoside and *p*NP-β-D-galactopyranoside. Therefore, this enzyme has high substrate specificity for the disaccharide glycone, as well as the aglycone moiety. Similarly, β-primeverosidase was able to hydrolyze other compounds that have disaccharide moieties, such as β-visianoside, furcatin, and β-gentiobioside, but cannot efficiently hydrolyze monoglycosides (*p*NP-Glu and *p*NP-Xyl) [2]. In addition, its relative activity toward its natural substrate β-primeveroside was much higher than that toward the other natural disaccharide glycosides tested. Thus, furcatin hydrolase and β-primeverosidase are examples of unique disaccharide specific glycosidases, in that they showed highest specificity for their natural substrates, furcatin and β-primeverosides, respectively.

Hösel and Barz [11] isolated β-glucosidases from garbanzo bean (*Cicer arietinum* L.) roots, leaves, and hypocotyls that were specific for isoflavone 7-*O*-glucosides. These β-glucosidases had the greatest affinity for isoflavone 7-*O*-β-D-glucosides and were highly specific for the 7-position of the aglycone in isoflavonoid and flavonoid glycosides. In addition, these enzymes also removed the β-1,6-apiosyl-glucose of biochanin A 7-*O*-β-D-glucoapioside as a disaccharide, which was not further hydrolyzed, indicating they also act as diglycosidases.

Previously, we reported the purification of a β-glucosidase and its natural substrates from *Dalbergia nigrescens* Kurz seeds [12]. This *D. nigrescens* β-glucosidase (Dnbglu) was similar to *Dalbergia cochinchinensis* β-glucosidase (dalcochinase, also called Thai rosewood β-glucosidase) in its activity toward a variety of synthetic glycoside substrates [13]. However, unlike dalcochinase, Dnbglu had high activity toward its diglycoside natural substrates dalpatein 7-*O*-β-D-apiofuranosyl-(1 → 6)-β-D-glucopyranoside (II) and dalnigrein 7-*O*-β-D-apiofuranosyl-(1 → 6)-β-D-glucopyranoside (III) (Fig. 1), releasing β-1,6-apiosyl-glucose disaccharide and aglycones [12]. *D. nigrescens* β-glucosidase was better than dalcochinase at hydrolyzing isoflavonoid β-glucosides from soybean, such as genistin (V) and daidzin (VI), as well as six-conjugated glycosides, such as malonyl genistin [14], but poorer at hydrolyzing dalcochinin β-D-glucopyranoside (I), the natural substrate of the *D. cochinchinensis* β-glucosidase [12]. Sequencing of the N-terminus and four isolated peptides from Dnbglu indicated that its sequence is quite similar to that of dalcochinase, despite their functional differences.

In order to understand the structural basis of the activity differences between the closely related *Dalbergia* β-glucosidases, we cloned a *D. nigrescens* β-glucosidase cDNA, *Dnbglu2*. Recombinant expression of Dnbglu2 and dalcochinase, in combination with mutagenesis of the latter allows us to more directly explore what differences in primary structure may explain their functional differences.

Materials and methods

Chemicals

Oligonucleotides were purchased from the BioService Unit of the National Science and Technology Development Agency of Thailand or from Proligo Inc. (Singapore). The pPICZαB plasmid and Superscript II reverse transcriptase were purchased from Invitrogen (Carlsbad, CA, USA) and *Taq* and *Pfu* polymerases from Promega (Madison, WI, USA). Restriction enzymes were from New England Biolabs (Beverly, MA, USA). Synthetic substrates, glucose-oxidase/peroxidase mixture (PGO), 2-mercaptoethanol, polyvinyl-polypyrolidone (PVPP), phenylmethylsulfonyl fluoride (PMSF), and Dowex 2 × 8 resin were obtained from Sigma Chemical (St. Louis, MO, USA). All other reagents for protein purification and characterization of enzyme kinetic were analytical grade or better.

Plant material and yeast strains

Dalbergia nigrescens seeds (mature and immature) were collected from a tree on the Suranaree University of Technology campus, Nakhon Ratchasima, Thailand. *Pichia pastoris* strain YM11430 was kindly provided by Dr. Joan Linn Cereghino, while strain GS115 was obtained from Invitrogen.

Purification of Dalbergia glycosidases and glycosides

The *D. nigrescens* glycosidase and glycosides were purified from *D. nigrescens* seeds as previously reported [12]. Dalcochinin β-D-glucoside (I) 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 I 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 I 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 ($[M+Na]^+ = 597.13$).

Mass spectrometric identification of tryptic peptides and their glycosylation

For the tryptic digest, 6 μg (0.1 nm) of Dnbglu purified from *D. nigrescens* seeds was dissolved in 40 μL of 100 mM NH_4HCO_3, mixed with 5 μL of 10 mM DTT, and incubated for 30 min at 50 °C. The solution was chilled, 5 μL of 100 mM iodoacetamide was added, and the solution was incubated in the dark at room temperature for 15 min. Then, 500 ng of sequence grade trypsin (Boehringer Mannheim, Germany) was added and incubated overnight at 37 °C. Trypsin-digested protein (0.08 nmol) was deglycosylated by incubation with 25 mU Endoglycosidase H (Roche, Mannheim, Germany) at 37 °C overnight. The sample was desalted and concentrated using GELoader tip microcolumns packed with Poros R2 material (Applied Biosystems, Framingham, MA). Analysis of N-linked glycosylation sites was performed by automated nanoflow liquid chromatography/tandem mass spectrometric analysis on an ESI QTOF Ultima mass spectrometer (Waters/Micromass UK Ltd., Manchester, UK). The nanoLC system was run with a linear gradient from Solution A (0.6% acetic acid) to 40% of solution B (80% acetonitrile, 0.5% acetic acid) in 90 min. The mass spectrometer was operated in the positive ion mode with a resolution of 9000–11,000 full-width half-maximum (FWHM) using a

source temperature of 80 °C and a counter current nitrogen flow rate of 150 L/h. Data dependent analysis was employed (the four most abundant ions in each cycle were subjected to MS/MS): 1 s MS (m/z 350–1500), 4×1 s MS/MS (m/z 50–2000, continuum mode), and 30 s dynamic exclusion. Raw data were processed using ProteinLynx Global Server ProteinLynx 2.0.5 (smooth 5/3 Savitzky Golay and center 4 channels/80% centroid) and the resulting MS/MS data set exported in the Micromass.pkl format. The spectral data were searched against Viridiplantae (green plants) in the NCBI database with Mascot software (Matrix Sciences Ltd., Cheshire, UK).

MALDI-MS of the intact protein was performed on a Voyager STR (PerSeptive Biosystems, Framingham, MA) equipped with delayed extraction. Spectra were obtained in positive reflector or linear ion mode, and negative reflector ion mode, with an accelerating voltage of 20 kV.

Cloning of the Dnbglu2 cDNA

The total RNA was isolated from immature seeds collected from a *D. nigrescens* tree 2–3 months after flowering. Typically, 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 [16], 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. [16]. 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 (CACCATTTgTAACCATTTTTCATTg) and PBGrev2 (gTAAAgCAACAAATCTCgAAgTCC), 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 [17]. 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 was done with *Taq* polymerase and the Q_o and PTDnF1 (ggTggCTTCTTAgATCgTAg) primers. 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 (gCCATTTgTggTgAAgACTTg) 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 with *Taq* polymerase, and the Q_T and PTDnR1 primers. A second amplification was performed with the product and the PTDnR2 (CgAAAATCATTTACAACCCTAC) 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 (AAATgTACCAAAgCCACAAAC) and Dn2_5′UTRf1 (TCCTTTCTTTCATCTCATgATTg) primers and cloned and sequenced as described above.

Sequence analysis and homology modeling

Initial analysis of DNA sequences was done with BLAST [19] at the National Center for Biotechnology Information (NCBI: http://www.ncbi.nlm.nih.gov). The N-terminal signal sequence was predicted with SignalP [20] 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 [21,22]. 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 and the Protpars parsimony program of the PHYLYP package [23]. Homology models were produced by alignment of the sequences of the *Dalbergia* β-glucosidases with the 1CBG structure in the Swiss PDBViewer, followed by submission to SwissModel for optimized model building [24]. The active sites were superimposed and illustrated by ray tracing with the PovRay program.

Expression vector construction

The DnVPPFPstI (gTgCAACCATTCCTgCAgTTCCTCCATTCA ATCgAAg) and Dn2CTERMXbaIr (ATCAAAATgCTTgAATggCCC ACTT) primers were used in PCR to introduce the desired PstI 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 [18] and an XbaI 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 PstI and XbaI, and the insert was gel purified and cloned into the pPICZαNH$_8$ plasmid [18].

Mutagenesis of dalcochinase

The site directed mutagenesis of dalcochinase residues A454 to S, E455 to G, and A454/E455 to SG was performed with a QuikChange Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA, USA). The two primer pairs used to introduce the single mutations were DcA454Sf (ggACA ACTTTgAATggTCTgAgggTTATACATCACgATTTg) and DcA454Sr (CAAATCgTgATgTATAACCCTCAgACCATTCAAAgTTgTCC) for A454S, and DcE455Gf (ggACAACTTTgAATgggCTgggggTTATACAT CACgATTTg) and DcE455Gr (CAAATCgTgATgTATAACCCCCAgC CCATTCAAAgTTgTCC) for E454G and the pPICZ-NH$_8$-trncTRBG clone [18] was used as the template. The double point mutation was generated by the same method with the A454S mutant as template and the Dc454/5SGf (ggACAACTTTgAATggTCTgggggTTATACATCACgATT Tg) and Dc454/5SGr (CAAATCgTgATgTATAACCCCCAgACCATTC AAAgTTgTCC) primers. The mutated plasmid sequences were checked by DNA sequencing.

Recombinant expression and purification of Dnbglu2 and wildtype and mutant dalcochinase

The selected plasmid clones were linearized with SacI 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*NP-Fuc 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. [25]. The media were 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).

Determination of substrate specificity

To determine the kinetic properties of dalcochinase from seed and Dnbglu2 from *P. pastoris* media toward natural *D. nigrescens* substrates, the aglycone products were quantified by HPLC. The reactions were performed by incubating dalpatein 7-*O*-β-D-apiofuranosyl-(1,6)-β-D-glycopyranoside (II) and dalnigrein 7-*O*-β-D-apiofuranosyl-(1,6)-β-D-glucopyroside (III) glycosides at various concentrations with 1.5 U of dalcochinase from seed and 0.01 U of Dnbglu2 β-glucosidase in 50 μL of 50 mM sodium acetate, pH 5.5, at 30 °C for 10 min. The reactions were stopped by boiling for 5 min. The reactions were dried and resuspended in 50 μL of methanol, and then 20 μL of each sample was injected for HPLC analysis.

HPLC was performed on an Eclipse XDB-C18 (4.6 × 250 mm, 5 μm) reverse phase column on an HP-Series 1100 HPLC (Agilent Corp., Palo Alto, CA, USA) at a flow rate of 0.8 mL/min at 30 °C. The column was equilibrated with aqueous 0.1% TFA. After the sample was injected, methanol was increased to 100% in a linear gradient from 0 to 20 min. The aglycone products were detected by measuring the absorbance at 320 nm. A standard curve of the aglycone of each glycoside substrate was produced under the same conditions over the range of 0.025–0.4 mM. Kinetic parameters were generated from nonlinear regression of the Michaelis–Menten curves and linear regression of [S]/v vs. [S] (Hanes) plots with Grafit 5.0 (Erithacus Software, Horley, UK).

The kinetics properties of the mutant dalcochinases and Dnbglu2 toward *p*NP-Glu and *p*NP-Fuc were determined by incubating approximately 0.1 pmol of mutant dalcochinase or Dnbglu2 with 0.4–18 mM *p*NP-Glu and 0.4–15 mM *p*NP-Fuc in 70 μL of 50 mM sodium acetate, pH 5.5, at 30 °C for 10 min. The reactions were stopped by adding 2 volumes of 2 M Na_2CO_3 and their absorbance at 405 nm was measured. The amount of released *p*NP was calculated from a *p*NP standard curve ranging from 0 to 100 μmol. One unit of enzyme is defined as the amount of enzyme releasing 1 μmol *p*NP per minute from 1 mM *p*NP-Glu at 30 °C. The kinetic parameters with standard errors were calculated from nonlinear regression of the Michaelis–Menten equation curves with the Enzfitter 1.05 program (Elsevier Biosoft, Cambridge, UK). Values were compared with those from linear regression of the [S]/v vs. [S] curves for *p*NP-Glu, to validate the use of nonlinear regression where saturation curves could not be obtained due to substrate solubility.

Comparison of relative levels of hydrolysis of isoflavonoid glycosides

To evaluate the hydrolysis of I, II, and III by *Dalbergia* β-glucosidases, 0.01 U of an enzyme was incubated with 50 μL of 1 mM substrate at 30 °C for 16 h. The reaction was stopped by boiling 5 min, dried and resuspended in 5 μL of methanol. The released aglycones were analyzed on analytical Silica gel 60, F_{254} aluminum TLC sheets with $CHCl_3$/MeOH/water (7.5:1:0.5) (v/v) solvent and UV absorbent spots were visualized under a UV box and compared with undigested glycosides. The plates were then sprayed with 10% sulfuric acid and heated at 110 °C for approximately 10 min to detect sugars.

The relative activities of Dnbglu2 and wildtype and mutant dalcochinases toward I and isoflavonoid glycosides from soybean were also compared by measuring the amount of glucose released. In 50-μL reactions, 0.001 U of wildtype dalcochinase and 0.005 U of Dnbglu2 and mutant dalcochinases were incubated with 1 mM I in 0.1 M sodium acetate, pH 5.0, at 30 °C for 10 min. The reactions were stopped by boiling 5 min. One hundred microliters of PGO and 50 μL of ABTS (1 mg/mL) were added, mixed and incubated at 37 °C for 30 min. The reactions were transferred to a microtiter plate and the absorbance was measured at 405 nm. Similarly, 0.001 U of wildtype and mutant dalcochinases and Dnbglu2 were incubated with 50 μL of 1 mM daidzin or genistin for 10 min at 37 °C. The reactions were stopped by boiling 5 min and 5 μL of the reaction was taken to assay with glucose oxidase, as described above.

Results

Hydrolysis of D. nigrescens disaccharide glycosides by D. cochinchinensis dalcochinase

Initial attempts to hydrolyze dalpatein 7-*O*-β-D-apiofuranosyl-(1,6)-*O*-β-D-glucopyranoside (II) and dalnigrein 7-*O*-β-D-apiofuranosyl-(1,6)-*O*-β-D-glucopyranoside (III), natural substrates of Dnbglu, with dalcochinase did not show any hydrolysis. However, when the amount of enzyme was increased, hydrolysis could be detected on TLC, and the sugar product comigrated with 6-apiosyl-β-D-glucose (acuminose) produced by digestion of the substrates with Dnbglu, indicating that the dalcochinase has isoflavonoid 7-*O*-apiosylglucosidase (E.C. 3.2.1.161, acuminosidase) activity, though at a low level (results not shown). Dalcochinase had an apparent K_m of 4.3 mM, k_{cat} of 0.2 s^{-1}, and k_{cat}/K_m of 47 M^{-1} s^{-1} for II and an apparent K_m of 10 mM, k_{cat} of 0.5 s^{-1}, and k_{cat}/K_m of 49 M^{-1} s^{-1} for III. This compares with a K_m of 0.5 mM, k_{cat} of 465 s^{-1}, and k_{cat}/K_m of 9.9×10^5 M^{-1} s^{-1} for II and a K_m of 0.7 mM, k_{cat} of 334 s^{-1}, and k_{cat}/K_m of 4.8×10^5 M^{-1} s^{-1} for III hydrolysis by Dnbglu purified from *D. nigrescens* seeds [12], indicating Dnbglu is 21,000-fold more efficient in hydrolyzing II and 9800-fold more efficient in hydrolyzing III compared to dalcochinase.

cDNA cloning and sequence analysis

In order to determine the primary sequence of Dnbglu and identify the amino acid residues that may account for the functional differences between the two closely related *Dalbergia* β-glucosidases, cDNA were cloned from *D. nigrescens* seeds with PCR primers designed from the *D. cochinchinensis* β-glucosidase cDNA sequence [16]. RNA from immature seeds and germinating seeds were used as templates. Specific primers designed from the initial partial cDNA clones were used to clone the *Dnbglu2* cDNA (GenBank Accession No. AF163097).

The full-length *Dnbglu2* cDNA sequence consisted of 1964 nucleotides, which included a 1593-nucleotide ORF encoding a 531 amino acid precursor protein (Fig. 2). The Dnbglu2 protein was predicted to contain a 23 amino acid signal sequence by the SignalP program [20], giving 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] (Fig. 2). However, N-terminal sequencing of Dnbglu purified from seeds previously showed the sequence A-T-I-T-E-V [12], which occurs eight 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 deg-

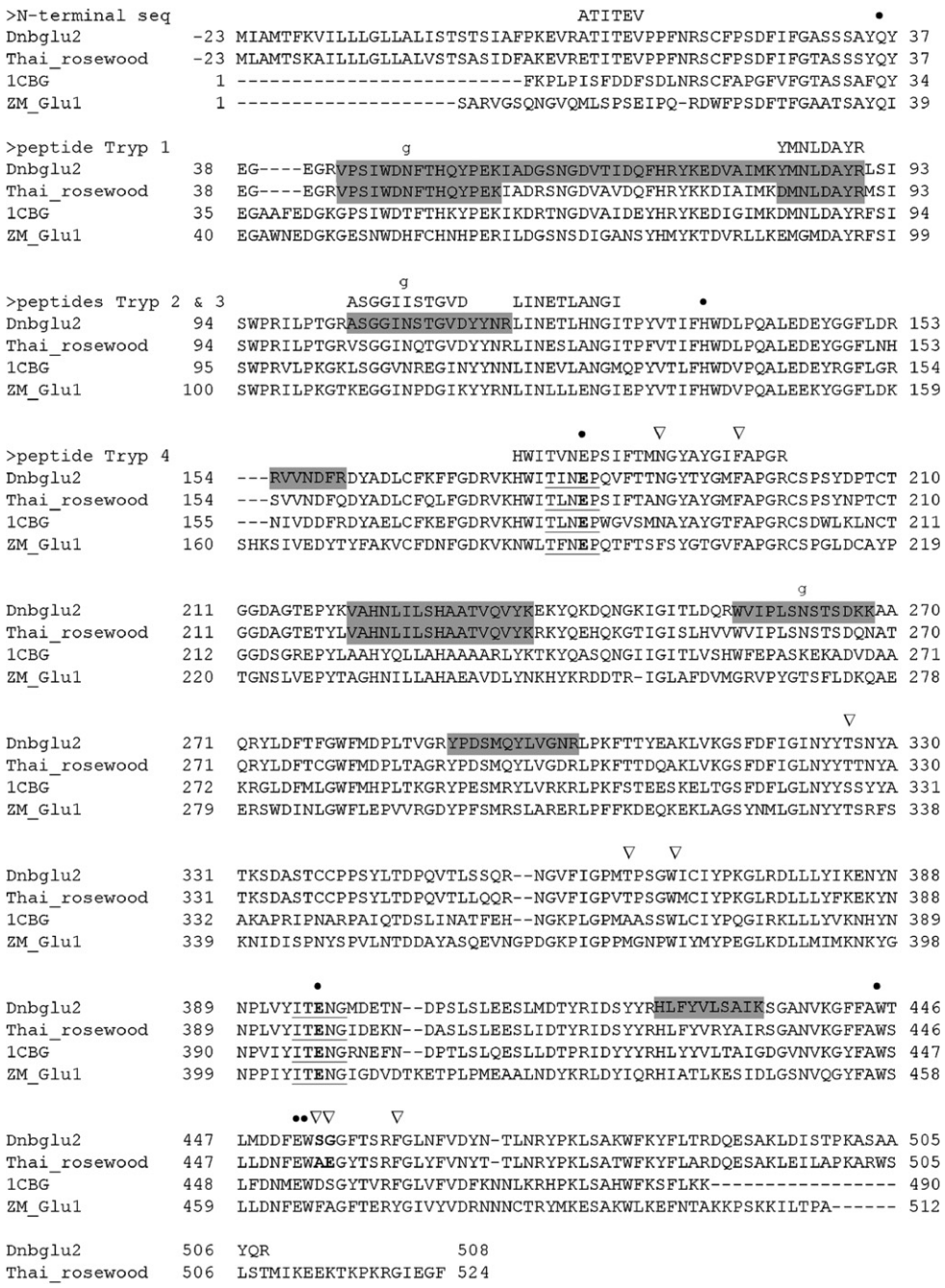


Fig. 2. Protein sequence alignment of β-glucosidases from *D. nigrescens*, Dnbglu2; *D. cochinchinensis* (dalcochinase, Dcbglu), cyanogenic β-glucosidase from white clover, (PDB: 1CBG) and *Zea maize* β-glucosidase 1 (ZMGlu1). N-terminal and tryptic peptide sequences obtained by Edman degradation of the purified protein are aligned above the full sequences [12]. Peptide sequences matching the masses of tryptic peptides in LCMS are shown with gray background. Sites shown to be glycosylated by LC/MS/MS are denoted with a small g above the glycosylated N. The sites indicated by Czjzek et al. [28] as interacting with the glycone (●) or aglycone (∇) of maize β-glucosidase are marked and the catalytic acid/base and nucleophile consensus sequences are underlined with the corresponding glutamates in bold. The single point and double point mutations of recombinant of *D. cochinchinensis* are in bold without underline.

radation 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 Fig. 2, 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.

The predicted Dnbglu2 protein sequence is slightly different from the four peptide sequences previously determined from β-glucosidase purified from *D. nigrescens* seeds [12] (Fig. 2). As noted, the chemically sequenced N-terminus matched the Dnbglu2 sequence, but the predicted N-terminus preceded it by eight 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 7th 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 nano-LC/MS/MS was used to further characterize the Dnbglu purified from seeds, 10 tryptic peptides with masses corresponding to Dnbglu2 were identified, as shown by the underlined regions in Fig. 2 and listed in Table 1. Though some of these peptides are identical to the corresponding peptides from dalcochinase, RVVNDFR, IADGS NGDVTIDQFHR, WVIPLSNSTSDKK, YPDSMQYLV GNR, and HLFYVLSAIK, which correspond to the *m/z* 453.29, 582.34, 560.02, 750.43, and 595.91 ($[M+H]^{2+}$), respectively (Table 1 and Fig. 1), are unique to Dnbglu2 and confirm the presence of Dnbglu2 in the enzyme purified from plant. However, the mass for one peptide that corresponds to residues 83–90: DMNLDAYR of dalcochinase and did not match Dnbglu2 was also identified: 996.56 amu (data not shown). So, the enzyme from *D. nigrescens* seeds appears to be a mixture of isozymes, though Dnbglu2 is likely prominent. In fact, application of the purified protein to a QAE ion exchange column and elution with a slow gradient revealed two closely spaced peaks, but these could not be clearly distinguished by LC/MS/MS analysis of tryptic peptides (data not shown).

The Dnbglu2 protein sequence has five possible N-linked glycosylation sites, so the mature protein produced in the plant would be expected to be larger than the predicted values due to glycosylation. When the protein purified from the plant was analyzed by MALDI-MS, the ion peak was seen at 62,668 *m/z*, larger than the predicted mass of the protein and consistent with glycosylation. Analysis of endoglycosidase-F-treated tryptic peptides from the purified protein by nano-LC/MS/MS, identified three peptides matching predicted Dnbglu2 peptides plus *N*-acetyl hexosamine (*N*-HexNAc), which indicates *N*-acetyl glycosamine at N-linked glycosylation sites, as shown in Table 1. These were 1667.0 amu, which corresponds to residues 285–297 in the Dnbglu2 precursor: WVIPLSNSTSDKK plus N-HexNAc; 1776.0 amu, which corresponds to residues 132–146: ASGGINSTGVDYYNR plus *N*-HexNAc; and 2063.2 amu, which corresponds to residues 72–86: VPSIWDNFTHQYPEK plus *N*-HexNAc. The sequences of these peptides were confirmed by MS/MS sequencing, thereby showing that at least three of the five putative glycosylation sites in Dnbglu2 are glycosylated in the plant.

Dnbglu2 was compared to related β-glucosidases that had been previously studied by amino acid sequence alignment and was most similar to dalcochinase with 81% identity (Figs. 2 and 3). Phylogenetic analysis of these proteins showed that they are most closely related to legume isoflavonoid β-glucosidases and cyanogenic β-glucosidases, with a more distant relationship to the disaccharidases primeverosidase and furcatin hydrolase (Fig. 3). These enzymes group together and are more distant to other plant glycosyl hydrolase family 1 enzymes, such as rice BGlu1 and maize Glu1. Interestingly, the cyanogenic β-glucosidases from clover and cherry group more closely with the other legume β-glucosidases including soybean isoflavone conjugate β-glucosidase [26] than do the *Dalbergia* isofl-

Table 1
Identification of peptides and glycosylation sites in the mass fingerprint of β-glucosidase purified from *D. nigrescens* seeds and digested with trypsin and endoglycosidase H

Observed	M_r (expt)	M_r (calc)	Score	Expect	Peptide
453.293100	904.571648	904.487839	25	5.2	RVVNDFR
523.294700	1044.574848	1044.469833	16	36	YMNLDAYR
548.846200	1095.677848	1095.563385	53	0.0066	YKEDVAIMK
577.361200	1152.707848	1152.584839	(21)	15	YKEDVAIMK + carbamidomethyl (N-term)
595.914200	1189.813848	1189.685867	70	0.00023	HLFYVLSAIK
750.432700	1498.850848	1498.687408	15	54	YPDSMQYLVGNR + carbamidomethyl (N-term)
775.475200	1548.935848	1548.767120	16	58	WVIPLSNSTSDK + *N*-HexNac (Asn)
560.015300	1677.024072	1676.862076	41	0.21	WVIPLSNSTSDKK + *N*-HexNac (Asn)
839.529400	1677.044248	1676.862076	(16)	76	WVIPLSNSTSDKK + *N*-HexNac (Asn)
582.335900	1743.985872	1743.817566	39	0.27	IADGSNGDVTIDQFHR
888.996500	1775.978448	1775.796143	48	0.036	ASGGINSTGVDYYNR + *N*-HexNac (Asn)
622.082100	1863.224472	1863.036591	67	0.00068	VAHNLLLSHAATVQVYK
688.726300	2063.157072	2062.963577	56	0.0098	VPSIWDNFTHQYPEK + *N*-HexNac (Asn)

Peptides were identified from single run MS and MS/MS data by searching against Viridiplantae (green plants) of NCBI database using the Mascot software.

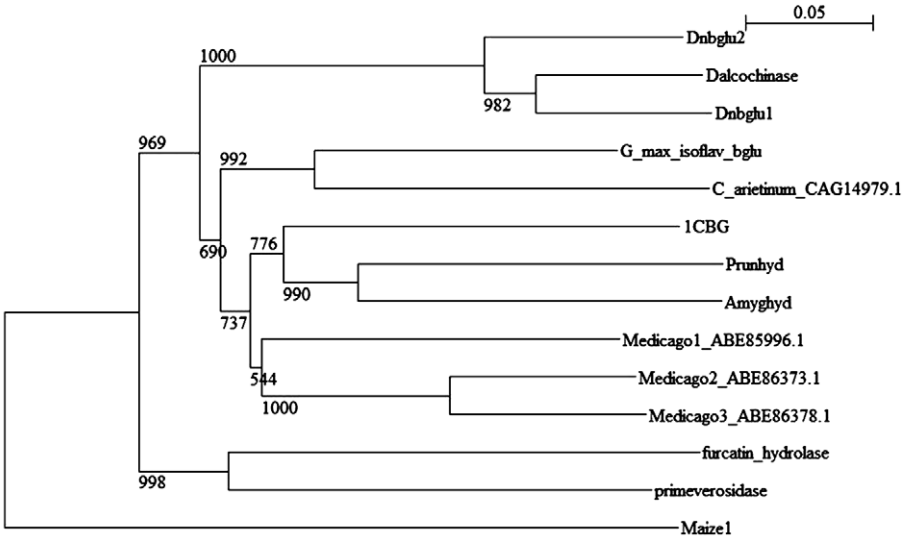


Fig. 3. Phylogenetic tree of *Dalbergia* isoflavonoid β-glucosidases with legume β-glucosidases and disaccharidases. The Maize Glu1 β-glucosidase (Maize1, Accession No. AAB03266) is included as an outgroup. The tree shows that the *Dalbergia* isoflavonoid β-glucosidases (*D. nigrescens* Dnbglu2 and *D. 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, [27]) and cherry (*Prunus serotina* Prunhyd, prunacin hydrolase, Accession No. 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 [6]. 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.

avonoid β-glucosidases, which fall in a somewhat more distantly related cluster. Thus, it would appear that the isoflavonoid β-glucosidases, which hydrolyze both glucosides and glycosides with 6-O-modified glucose residues, are derived from the same branch of GH1 as the defense-related glucosidases from other plants, and are more closely related to these than they are to the nonisoflavonoid diglycosidases.

Dnglu2 contains the catalytic acid/base E182 located in the consensus motif TINEP found in GH1 β-glucosidases, while the catalytic nucleophile residue E396 occurs within the sequence YITENG [27]. Czjzek et al. [28] identified four amino acid residues, W378, F198, F205, and F466 of maize β-glucosidase, which contact the substrate aglycone and were proposed to determine aglycone binding specificity. These amino acid residues correspond to W368, N189, F196, and S454 and A454 in Dnbglu2 and dalcochinase, respectively. An additional four residues of maize β-glucosidase were suggested to be involved in aglycone binding, T334, M374, Y473, and A467, which are equivalent to T326, T364, F461, and G455 and E455 in Dnbglu2 and dalcochinase, respectively. Homology modeling based on the white clover cyanogenic β-glucosidase structure (PDB: 1CBG, [27]), which is most closely related to Dnbglu2 among enzyme with known structures, confirmed that these residues are likely to be in the active site. Of these active site cleft residues, residues 454 and 455 show differences between the *Dalbergia* β-glucosidases and may thus help to determine the differences in substrate specificity between these enzymes (Fig. 4). Residues Q38, H142, E191, E406, E464, and W465 of maize β-glucosidase, which are highly conserved in GH1 β-glucosidases and are thought to be critical for glucose binding [28], correspond to Q36, H136, E182, E396, E452, and W453, respectively, in Dnbglu2 and dalcochinase. The interaction of the sugars by hydrogen bonds between the sugar hydroxyls and polar side chains, as well as van der Waals interactions between the alkyl hydrogens of sugars and aromatic residues at the sugar binding site of proteins, has been suggested to provide binding affinity and specificity to the interaction [29].

Recombinant expression of Dnbglu2

When recombinant *Dnblu2* was expressed in fusion with yeast α-factor prepropeptide in *P. pastoris*, increased *p*NP-Fuc hydrolase activity appeared in the media, and an approximately 63 kDa protein could be purified from desalted media by adsorption to cobalt chelating resin (IMAC). 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 four subunits, as are the native β-glucosidases purified from *D. cochinchinensis* and *D. nigrescens* seeds [13,12]. Recombi-

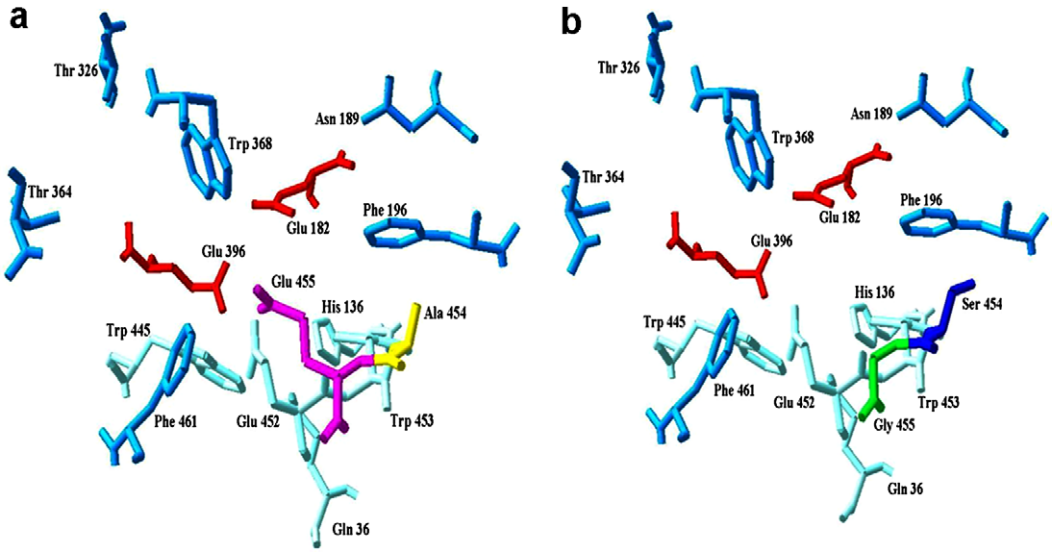


Fig. 4. Models of the active sites of dalcochinase (a) and Dnbglu2 (b) β-glucosidases. The models illustrate some differences between the *Dalbergia* β-glucosidases. Residues Ala454 (purple) and Glu455 (yellow) of Thai rosewood correspond to Ser454 (purple) and Gly455 (yellow) in Dnbglu2. The amino acid residues of the aglycone and glycone binding sites are in blue and light blue, respectively. The catalytic amino acid residues (Glu182 and Glu396) are indicated in red. The figure was produced with Pymol (DeLano scientific LLC).

nant dalcochinase was expressed and purified in the same way from the previously described construct [18].

Kinetic evaluation of Dalbergia β-glucosidases

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 2). The recombinant dalcochinase gave lower relative activity for *p*NP-β-D-galactoside, but showed higher relative activity toward *p*NP-β-D-mannoside compared to dalcochinase from seeds. Dnbglu2 had K_m values for both *p*NP-Glu and *p*NP-Fuc similar to those of natural *D. nigrescens* β-glucosidase from seeds (Table 3, [12]). Similarly, the wildtype recombinant dalcochinase had K_m values for both *p*NP-Glu and *p*NP-Fuc similar to dalcochinase from seeds, as previously reported [18].

The activity of the recombinant Dnbglu2 was also studied with its own natural substrates from *D. nigrescens* Kurz seeds and with I, the natural substrate from *D. cochinchinensis* seeds. In contrast to Dnbglu purified from seed [12], Dnbglu2 hydrolyzed III better than II with apparent K_m, k_{cat}, and k_{cat}/K_m values of 3.8 mM, 20 s^{-1}, and 5.32 mM^{-1} s^{-1} for II and 4.0 mM, 229 s^{-1} and 58.6 $mM^{-1}s^{-1}$ for III. The recombinant enzyme was less efficient at hydrolysis of both of these substrates compared to Dnbglu from seed, with K_m values 5- to 10-fold higher and a k_{cat} for II 20-fold lower, though the k_{cat} for III was similar. Nonetheless, Dnbglu2 maintains a high preference for II and III over I, which has a similar apparent K_m of 5.3 mM, but a much lower apparent k_{cat}, 0.049 s^{-1}, and

Table 2
Hydrolysis of glycoside substrates with recombinant *D. nigrescens* β-glucosidase compared with *D. nigrescens* β-glucosidase purified from seed and *D. cochinchinensis* β-glucosidase

Substrates	% Relative activity			
	Natural Dnbglu	Dnbglu2	Natural *D. cochinchinensis*	Wildtype *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.
Assays were done with 5 mM substrate for 10 min under standard assay conditions.

Table 3
Kinetics of recombinant β-glucosidases toward *p*NP-glucoside and *p*NP-fucoside

Recombinant enzymes	*p*NP-Glucoside			*p*NP-Fucoside		
	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m (s^{-1} M^{-1})	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m (s^{-1} M^{-1})
Dnbglu2	26.1 ± 2.6	298 ± 19	$(1.14 \pm 0.19) \times 10^4$	1.67 ± 0.08	92.8 ± 1.2	$(5.57 \pm 0.32) \times 10^4$
Dalcochinase	8.35 ± 0.51	174.6 ± 4.5	$(2.09 \pm 0.18) \times 10^4$	$(7.87 \pm 0.34) \times 10^{-1}$	96.03 ± 0.92	$(1.220 \pm 0.064) \times 10^5$
A454S Dalcochinase	5.89 ± 0.51	128.1 ± 4.1	$(2.18 \pm 0.26) \times 10^4$	$(4.74 \pm 0.42) \times 10^{-1}$	63.0 ± 1.0	$(1.33 \pm 0.14) \times 10^5$
E455G Dalcochinase	5.26 ± 0.22	158.9 ± 2.4	$(3.04 \pm 0.17) \times 10^4$	$(5.30 \pm 0.46) \times 10^{-1}$	(81.0 ± 1.4)	$(1.53 \pm 0.16) \times 10^5$
Dalcochinase double mutant	6.82 ± 0.53	281.4 ± 7.7	$(4.13 \pm 0.43) \times 10^4$	$(5.93 \pm 0.48) \times 10^{-1}$	103.6 ± 1.7	$(1.75 \pm 0.17) \times 10^5$

All recombinant were expressed in *Pichia pastoris* strain Y11430. Enzymes listed are as follows: dalcochinase, wildtype *D. cochinchinensis* β-glucosidase; dalcochinase A454S, A454S mutant of dalcochinase; dalcochinase E455G, E455G mutant of dalcochinase; dalcochinase double mutant, A454S/E455G mutant of dalcochinase; and Dnbglu2 from *D. nigrescens*. The apparent kinetic parameters with standard errors were obtained from nonlinear regression of the Michaelis–Menten curves with Enzfitter 1.05 (Elsevier Biosoft, Cambridge, UK).

k_{cat}/K_m, 0.0094 mM^{-1} s^{-1}. In contrast, the wildtype recombinant dalcochinase showed much less efficient hydrolysis of *D. nigrescens* substrates, as expected (Table 4).

The hydrolysis of I, II, and III by recombinant dalcochinase and Dnbglu2 was also compared by TLC (Fig. 5). The TLC confirmed that Dnbglu2 glucosidase could hydrolyze its natural substrates and release a disaccharide unit as does the β-glucosidase purified from *D. nigrescens* seeds [12], but it hydrolyzed I less efficiently. The recombinant dalcochinase was much more efficient at hydrolysis of I, and also hydrolyzed both natural substrates of *D. nigrescens*, although poorly. The sugar spots on the TLC indicated the disaccharide unit of β-D-apiofuranosyl-β-D-glucopyranose was released from II and III and had mobility similar to the standard disaccharide. No glucose or apiose was observed.

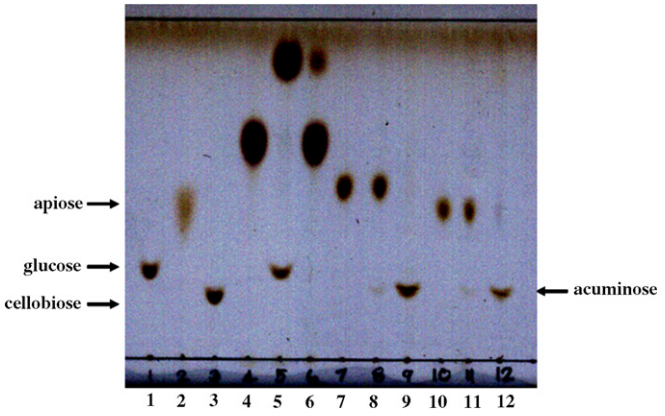


Fig. 5. TLC of I, II, and III hydrolysis by recombinant *D. nigrescens* Dnbglu2 and *D. cochinchinensis* dalcochinase. Digests were done with 0.01 U for 16 h at 30 °C. Lane 1, glucose std; lane 2, apiose std; lane 3, cellobiose std; lane 4, I std; lane 5, I hydrolyzed with recombinant *D. cochinchinensis*; 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 Fig. 1.

Mutagenesis of D. cochinchinensis β-glucosidase to D. nigrescens β-glucosidase

Since protein sequence alignment and homology modeling identified residues 454 and 455 as the residues in the substrate binding site that differ between dalcochinase and Dnbglu2, these positions in dalcochinase were mutated to the amino acids found in Dnbglu2 to see if the activity could be converted to that of the latter enzyme. Although Dnbglu2 has a 3-fold higher K_m for *p*NP-Glu and *p*NP-Fuc than dalcochinase, the effects on hydrolysis of *p*NP-Glu and *p*NP-Fuc of the A454S and E454G mutants were small and appeared to compensate each other in the double

Table 4
Rates of hydrolysis of isoflavonoid glycosides relative to wildtype dalcochinase

Enzyme	Isoflavonoid substrate				
	I	II	III	Daidzin (VI)	Genistin (V)
Wildtype dalcochinase	1.0	1.0	1.0	1.0	1.0
Dalcochinase A454S	0.94	1.4	0.44	0.99	0.88
Dalcochinase E455G	0.89	1.4	0.40	1.3	1.2
Dalcochinase double mutant	1.1	4.0	6.8	1.3	2.0
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 are given relative to wildtype dalcochinase. Enzymes listed are as follows: Dnbglu2 from *D. nigrescens*, wildtype dalcochinase, *D. cochinchinensis* dalcochinase; dalcochinase A454S, A454S mutant of dalcochinase; dalcochinase E455G, E455G mutant of dalcochinase; and dalcochinase double mutant, A454S/E455G mutant of dalcochinase.

mutant (Table 3). When the mutant enzymes were tested for hydrolysis of natural substrates, all mutants maintained the ability to efficiently hydrolyze I (Table 4). The single mutants showed similar hydrolysis of II and half the rate of hydrolysis III compared to wildtype dalcochinase. However, the double mutant showed 4-fold more rapid hydrolysis of II and 7-fold more rapid hydrolysis of III compared to wildtype dalcochinase. The double mutant also had a 2-fold increase in the hydrolytic rate for genistin, but showed little increase for daidzin. Therefore, the double mutation of A454S/E455G had little effect on hydrolysis of *p*NP glycosides, but improved hydrolysis of the *D. nigrescens* isoflavonoid glycoside substrates in a manner that could not be explained by a combination of the effects of the individual mutations.

Discussion

β-Glucosidases that hydrolyze isoflavonoid 7-*O*-β-glucosides and their 6″ conjugates have been found in chickpea [11] and soybean [26], in addition to *D. nigrescens* [12]. We have described the primary structure and activity of such a β-glucosidase from *D. nigrescens*, Dnbglu2, in this paper and started to identify the basis for this unique activity for the first time. The recombinantly expressed Dnbglu2 was able to hydrolyze isoflavonoid 7-*O*-β-D-apiosyl-1,6-β-D-glucosides and malonyl glucosides, and to release the disaccharide from the former. The Dnbglu2 expressed in *Pichia pastoris* media appeared to be similar to the enzyme purified from *D. nigrescens* seeds, but was less efficient in hydrolysis of the II and III substrates and showed a few other differences, possibly because a mixture of isozymes was likely to have been present in the preparation from seeds, due to a sequence difference in the recombinant enzyme and the primary seed isozyme or due to differences in post-translational modification of this glycoprotein. Nonetheless, Dnglu2 showed 400- and 5000-fold higher k_{cat}/K_m values in hydrolyzing II and III, respectively, compared to I, which is similar to the Dnbglu purified from seed. The closely related dalcochinase purified from *D. cochinchinensis* seeds or expressed in *Pichia* was also found to have isoflavonoid β-D-apiosyl-1,6-β-D-glucosidase activity, but at a much lower level than Dnbglu from seeds and Dnbglu2. Mutagenesis showed that differences in two residues found in the substrate binding cleft partially accounted for the difference in activity toward these disaccharide glycoside substrates.

Among the amino acid residues identified by Czjzek et al. [28] to be part of the ligand binding site of maize ZmGlu1, only two residues differ between Dnbglu2 and dalcochinase. Neither the A454S nor the E455G mutation of dalcochinase gave a improvement of the hydrolysis of *D. nigrescens* isoflavonoid substrates, but the combination of these two mutants allowed substantial hydrolysis. Mutation of either of the two sites had only slightly positive effects on hydrolysis of II and a 2-fold decrease in hydrolysis of III, rather than the larger positive effect on hydrolysis seen in the double mutant. It could be surmised from the synergism of the double mutation that the effects of S454 and G455 are likely on the same reaction step and are completely cooperative, since the presence of either A454 or E455 completely negated this effect [30,31]. This is understandable for adjacent residues that likely interact. Perhaps the space created with the replacement of E455 with G allows a productive contact between the S454 hydroxyl and the isoflavonoid diglycosides.

Previously, Verdoucq et al. [32] did the corresponding double mutation of maize ZmGlu1 residues F466 and A467 to the corresponding residues in sorghum dhurrinase (S462 and S463). This mutation allowed hydrolysis of dhurrin at 3% of the level of sorghum dhurrinase, which is similar to the effect of the double mutation reported here. These residues are involved in dhurrin binding in the dhurrinase structure, where S462 makes an indirect hydrogen bond through a water molecule to the hydroxyl group on the phenol ring [33]. Interestingly, although the A454S/E455G double mutation significantly increased the ability to hydrolyze the *D. nigrescens* isoflavonoid substrates and had some effect on genistin hydrolysis, it did not affect the ability of dalcochinase to hydrolyze *p*NP-Glu and *p*NP-Fuc, despite a 3-fold difference in K_m between dalcochinase and Dnbglu2. Thus, it seems that other factors, such as the overall shape of the active site are perhaps more important for the subtle differences seen with other substrates.

Inspection of the substrate structures in Fig. 1 shows that relative position of the glycosidic bond to be hydrolyzed is much closer to the isoflavonoid moiety of II and III than that of I, the natural substrate of dalcochinase. Thus, Dnbglu2 must be optimized for binding the isoflavonoid moiety closer to the catalytic residues than in dalcochinase. The combined differences of S instead of A at residue 454 and G instead of E at residue 455 apparently help Dnbglu2 to bind the isoflavonoid ring in the proper position for hydrolysis of the 7-*O*-glycosyl ring more than when it is bound in this position by dalcochinase. However, the activity gained by the double mutation was only 2–3% of the activity of Dnbglu2 on *D. nigrescens* substrates, and the effect was less clear for soy isoflavonoids, for which the differences between the two *Dalbergia* enzymes are less pronounced. This may indicate that these differences help it to accommodate the larger glycone group found in II and III and conjugated isoflavonoid glycosides. Other differences in the active site shape are likely to contribute more, since Dnbglu2 is much more active toward these substrates than the dalcochinase double mutant. In principle differences in the −2 site, the position of the apiosyl residue 1,6-linked to the glucosyl residue, that allow for Dnbglu to better accommodate the larger glycone would appear to be good candidates to contribute to this. In fact, inspection of the dhurrin glucosyl residue in the 1vO3 sorghum dhurrinase structure shows that the 6-hydroxyl group points out toward the entry of the active site [33], so the apiosyl residue would be expected to sit in the entry cleft across from

or even against the aglycone, where it might interact with putative aglycone binding residues. However, the glucosyl O6 sits around 9–10 Å away from the position of S454, so any affects on apiosyl binding are likely to be indirect, and, unlike primeverosidase and furcatin hydrolase, Dnglu appears to hydrolyze isoflavone glucosides better than diglycosides [12], so such differences in the glycone site could be rather subtle.

Recently, Suzuki et al. [26] reported the characterization of a conjugated isoflavone glucoside-specific β-glucosidase from soybean roots. This enzyme preferred malonyl genistin and malonyl daidzin to genistin and daidzin, and hydrolyzed isoflavone glycosides with high efficiency, but could not hydrolyze other natural substrates, such as flavone glycosides. Given the similar substrate specificity, one might expect that the active sites of the soybean and Dnbglu2 would be similar. However, of the aglycone-binding residues identified from comparison to maize [28], Dnbglu2 residues N189, F196 T326, T364, S454, and G455 are replaced by G, T, S, A, F, and A, respectively, in the soybean enzyme, while only W368 and F461 are conserved. Though some of these differences are conservative, many are not, and Dnbglu2 is much more similar to dalcochinase at these positions than to the soybean protein. The soybean enzyme appears to be stricter in its specificity for soy isoflavone conjugates, which is hydrolyzes with very high efficiency, so it may have different binding requirements [26]. Nonetheless, it appears that even closely related enzymes may have different means of binding similar substrates.

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Nucleotide and Derived Amino Acid Sequences of the Cyanogenic Beta-Glucosidase (Linamarase) from Cassava (*Manihot esculenta* Crantz)

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ABSTRACT

Many isozymes of cassava cyanogenic β-glucosidase (linamarase) exist, but only one cDNA sequence (pCAS5) has been reported thus far. In order to study the structure-function relationships in this enzyme, the cDNA of cassava linamarase was cloned and sequenced. In this report, six different cDNA sequences of linamarase from cassava were cloned by reverse transcription-polymerase chain reaction (RT-PCR) using primers designed from the sequence of pCAS5. Nucleotide sequences of all six clones showed 98-99% sequence identity to the nucleotide sequence of pCAS5. Derived amino acid sequences from four cDNA clones showed 98-99% sequence identity to that of pCAS5, while the other two cDNA clones contained nucleotide sequences that led to premature termination.

Key words: beta-glucosidase, linamarase, cassava, nucleotide sequence, amino acid sequence

INTRODUCTION

Cassava (*Manihot esculenta* Crantz) is an important crop due to the large storage roots which are a staple carbohydrate source for many populations worldwide. Young cassava leaves are also consumed by humans or used as animal feeds (Yeoh, 1989). However, both leaves and roots of cassava are cyanogenic, posing a potential health hazard to consumers. Cyanogenesis in cassava involves two cyanogenic glucosides (linamarin and lotaustralin) and sequential action of two enzymes (β-glucosidase and hydroxynitrile lyase). Normally, the glucoside substrates and the enzymes are kept in separate compartments. Exposure of these two cyanogenic glucosides to β-glucosidase, following tissue damage, results in their hydrolysis to yield glucose and an aglycone, which is acetone cyanohydrin or butanone cyanohydrin. These cyanohydrins may be broken down spontaneously or enzymatically by α-hydroxynitrile lyase to produce hydrogen cyanide (Eksittikul and Chulavatanatol, 1988; Hughes *et al*., 1992). The cyanogenic β-glucosidase, or linamarase, which is responsible for the first step of cassava cyanogenesis, is present in many parts of the plant, including petioles, stem, leaves, peels and tuber cortex. Physiologically, the enzyme plays an important role as a host defense against biological predators. Furthermore, an *in vitro* study has discovered the unique ability of cassava linamarase in alkyl glucoside synthesis by transferring glucose to a large variety of alcohol acceptors, while β-glucosidases from Thai rosewood and almond could not (Svasti *et al*., 2003). This difference in enzymatic action may

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lie in slight variations in the amino acid sequences of β-glucosidases from various sources. So, it is of scientific interest to understand the molecular basis of enzymatic catalysis in cassava linamarase. To achieve this, cassava linamarase must be cloned and expressed as a recombinant protein, its nucleotide sequence mutated, and the effects of mutation characterized. These procedures will help to identify amino acid residues responsible for transglucosylation activity.

A number of studies have shown the existence of multiple forms of cassava linamarase (Eksittikul and Chulavatanatol, 1988; Yeoh, 1989), however, only a single cDNA clone of linamarase (pCAS5) from cassava has been isolated thus far (Hughes *et al*., 1992). The identity of other cassava linamarase isozymes has not been reported. In this study, we reported the isolation of six different cDNA clones of cassava linamarase, and the amino acid sequences derived from four full-length cDNA sequences. The predicted polypeptides were very similar to that of the previously reported cassava linamarase, so they can be reasonably assumed to be catalytically active in cassava.

MATERIALS AND METHODS

Total RNA was isolated from young cassava leaves (local varieties) using RNeasy extraction kit (QIAGEN, USA) and concentration estimated by absorbance at 260 nm. Total RNA (0.2-5 μg) was converted into single stranded first strand cDNA fragment using 200 U SuperScript II reverse transcriptase (Invitrogen, USA) in the reaction containing 0.5 μg oligo$(dT)_{12\text{-}18}$ primer, 10 nmol dNTP mix, and 10 mM DTT at 42 °C for 50 min and at 70 °C for 15 min. The RNA strand of the RNA:cDNA duplex was degraded by 2 U RNase H at 37 °C for 20 min. The 1 μL aliquot of the product was used as a template for PCR with 1 μM each of CVLMF2 (5' TTC TTC AGC TAT CAG GGA TGC 3') and CVLMR2 (5' TGC TAG ATC ATT GGA GCT TCA 3') primers, 1.5 mM $MgCl_2$, 50 mM KCl, 0.1% Triton X-100, 0.2 mM of each dNTP and 2.5 U Expand HiFi DNA polymerase (Boehringer, Germany) in a total volume of 50 μL. All PCR reactions were done using a 94 °C initial denaturation for 2 min, 29 cycles of 94 °C for 60 s, 45 °C for 30 s, and 72 °C for 2 min, and a 72 °C final elongation for 8.5 min. The primer sequences correspond to bases 7-27 (sense) and bases 1677-1697 (antisense), respectively, of the cassava linamarase cDNA clone pCAS5 reported previously (Hughes *et al*., 1992). The resulting RT-PCR product was gel-purified, ligated into pGEM-Teasy vector (Promega, USA) and transformed into *E. coli* DH5α.

The nucleotide sequence of cDNA clones were determined by automated DNA sequencing (ABI 373A DNA sequencer). The nucleotide sequences obtained were translated into amino acid sequences using EMBOSS Transeq program. Alignment of nucleotide and amino acid sequences were done using ClustalW version 1.82. The physical and chemical parameters of the derived amino acid sequences were performed using ProtParam program.

RESULTS AND DISCUSSION

Cloning of cDNA of cassava linamarase

Total RNA was extracted from young cassava leaves. Approximately 500 ng total RNA was used to synthesized the first strand cDNA. PCR amplification of the first strand cDNA yielded 1.7 kb PCR product (Figure 1), which was then ligated into pGEM-Teasy vector and transformed into *E. coli* DH5α. The nucleotide sequences of cloned RT-PCR products were determined by automatic sequencing, yielding six different cDNA clones, namely 2A.29, 2A.32, 2A.33, 2A.52, 2A.55 and 2A.62. Alignment of the nucleotide sequences from all six cDNA clones showed 98-99% sequence identity to the previously reported nucleotide sequence of the cassava linamarase

cDNA clone pCAS5 (Hughes *et al*., 1992), and to each other (results not shown).

Alignment of nucleotide and derived amino acid sequences of cassava linamarase

The nucleotide sequences from 6 cDNA clones were translated and aligned. A thymine deletion in clone 2A.29, corresponding to position 570 in pCAS5, resulted in a frame-shift mutation from that position onward and a pre-mature termination of 7 amino acids later. Also in clone 2A.33, an adenine-to-thymine substitution, corresponding to position 225 in pCAS5, resulted in a non-sense mutation at that position. So, only four cDNA clones, namely 2A.32, 2A.52, 2A.55 and 2A.62, encoded the full-length amino acid sequence of cassava linamarase. Alignment of the amino acid sequences derived from these four cDNA clones showed 98-99% sequence identity to the previously reported amino acid sequence of the cassava linamarase derived from pCAS5 (Hughes *et al*., 1992), and to each other (Figure 2). All four predicted polypeptides contained the highly conserved TFNEP and VTENG motifs bearing the catalytic acid/base (Glu198) and catalytic nucleophile (Glu413), respectively. The five putative N-glycosylation motifs, NX(S/T), were also conserved. The polypeptides derived from cDNA clones were analyzed for their physical and chemical parameters (Table 1).

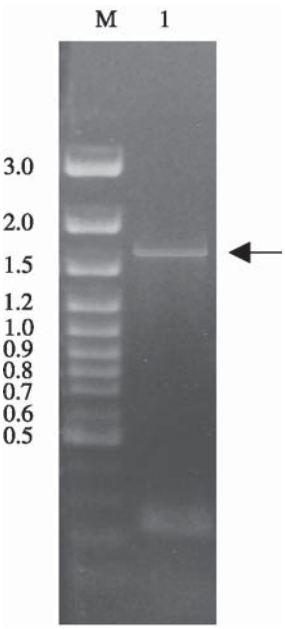

Figure 1 The cDNA of cassava linamarase (analyzed on 1% (w/v) agarose and stained with ethidium bromide). Lane 1, the 1.7 kb RT-PCR product (indicated by an arrow); M, DNA size marker (kb).

The cDNA sequences reported here were synthesized from young cassava leaves, which had been shown to be the site of cassava linamarase synthesis (Poncoro and Hughes, 1992). This is likely to produce different linamarase transcripts from the previously reported cassava linamarase cDNA sequence, pCAS5, which was extracted from yellow cotyledons (Hughes *et al*., 1992). The four polypeptides predicted from the four cDNA clones were very similar to the previously reported cassava linamarase, in terms of sequence identity, physico-chemical properties as well as the presence of the key motifs essential for catalytic activity. So, these four predicted polypeptides can

Table 1 Physical and chemical parameters of the polypeptides derived from four cDNA clones.

cDNA	Number of amino acids	Molecular weight	Theoretical p*I*
pCAS5	531	61,372.9	5.52
2A.32	531	61,461.0	5.44
2A.52	531	61,488.1	5.62
2A.55	531	61,323.8	5.44
2A.62	531	61,370.8	5.35

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pCAS5           MLVLFISLLALTRPAMGTDDDDDNIPDDFSRKYFPDDFIFGTATSAYQIEGEATAKGRAP 60
2A.32           MLVLFISLLVLTRPAMGTDDDDDNIPDDFSRKYFPDDFIFGTATSAYQIEGEATAKGRAP 60
2A.52           MLVLFISLLVLTRPAMGTDDDDDNIPDDFSRKYFPDDFIFGTATSAYQIKGEATAKGRAP 60
2A.55           MLVLFISLLALTRPAMGTDDDDDNIPDDFSRKYFPDDFIFGTATSAYQIEGEATAKGRAP 60
2A.62           MLVLFISLLALTKPAMGTDDDDDDIPGDFNRNYFPDDFIFGTATSAYQIEGEATAKGRAP 60
                *********.**:**********:**.**.*:*****************:**********

pCAS5           SVWDIFSKETPDRILDGSNGDVAVDFYNRYIQDIKNVKKMGFNAFRMSISWSRVIPSGRR 120
2A.32           SVWDIFSKETPDRILDGSNGDVAVDFYNRYIQDIKNVKKMGFNAFRMSISWSRVIPSGRK 120
2A.52           SVWDIFSKETPDRILDGSNGDVAVDFYNRYIQDIKNVKKMGFNAFRMSISWSRVIPSGRR 120
2A.55           SVWDIFSKETPDRILDGSNGDVAVDFYNRYIQDIKNVKKMGFNAFRMSISWSRVIPSGRR 120
2A.62           SVWDIFSKETPDRILDGSNGDVVVDFYNRYIQDIKNVKKMGFNAFRMSISWSRVIPSGRR 120
                **********************.************************************:

pCAS5           REGVNEEGIQFYNDVINEIISNGLEPFVTIFHWDTPQALQDKYGGFLSRDIVYDYLQYAD 180
2A.32           REGVNEEGIQFYNDVINEIISNGLEPFVTIFHWDTPQALQDKYGGFLSRDIVYDYLQYAD 180
2A.52           REGVNEEGIQFYNDVINEIISNGLEPFVTIFHWDTPQALQDKYGGFLSRDIVYDYLQYAD 180
2A.55           REGVNEEGIQFYNDVINEIISNGLEPFVTIFHWDTPQALQDKYGGFLSRDIVCDYLQYAD 180
2A.62           REGVNEEGIQFYNDVINEIISNGLEPFVTIFHWDTPQALQDKYGGFLSRDIVYDYLQYAD 180
                **************************************************_********

pCAS5           LLFERFGDRVKPWMTFNEPSAYVGFAHDDGVFAPGRCSSWVNRQCLAGDSATEPYIVAHN 240
2A.32           LLFERFGDRVKRWMTFNEPSAYVGFAHDDGVFAPGRCSSWVNRQCLAGDSATEPYIVAHN 240
2A.52           LLFERFGDRVKRWMTFNEPSAYVGFAHDDGVFAPGRCSSWVNRQCLAGDSATEPYIVAHN 240
2A.55           LLFERFGDRVKRWMTFNEPSAYVGFAHDDGVFAPGRCSSWVNRQCLAGDSATEPYIVAHN 240
2A.62           LLFERFGDRVKRWMTFNEPSAYVGFAHDDGVFAPGRCSSWVNRQCLAGDSATEPYIVAHN 240
                ***********_**************************************************

pCAS5           LLLSHAAAVHQYRKYYQGTQKGKIGITLFTFWYEPLSDSKVDVQAAKTALDFMFGLWMDP 300
2A.32           LLLSHAAAVHQYRKYYQGTQKGKIGITLFTFWYEPLSDSEVDVQAAKTALDFMFGLWMDP 300
2A.52           LLLSHAAAVHQYRKYYQGTQKGKIGITLFTFWYEPLSDSEVDVQAAKTALDFMFGLWMDP 300
2A.55           LLLSHAAAVHQYRKYYQGTQKGKIGITLFTFWYEPLSDSEVDVQAAKTALDFMFGLWMDP 300
2A.62           LLLSHAAAVHQYRKYYQGTQKGKIGITLFTFWYEPLSDSEVDVQAAKTALDFMFGLWMDP 300
                ****************************************:*********************

pCAS5           MTYGRYPRTMVDLAGDKLIGFTDEESQLLRGSYDFVGLQYYTAYYAEPIPPVDPKFRRYK 360
2A.32           MTYGRYPRTMVDLAGDKLIGFTDEESQLLRGSYDFVGLQYYTAYYAEPIPPVDPKFRRYK 360
2A.52           MTYGRYPRTMVDLAGDKLIGFTDEESQLLRGSYDFVGLQYYTAYYAEPIPPVDPRFRRYK 360
2A.55           MTYGRYPRTMVDLAGDKLIGFTDEESQLLRGSYDFVGLQYYTAYYAEPIPPVDPKFRRNK 360
2A.62           MTYGRYPRTMVDLAGDKLIGFTDEESQLLRGSYDFVGLQYYTAYYAEPIPPVDPKFRRYK 360
                **************************************************:***_*

pCAS5           TDSGVNATPYDLNGNLIGPQAYSSWFYIFPKGIRHFLNYTKDTYNDPVIYVTENGVDNYN 420
2A.32           TDSGVNATPYDLNGNLIGPQAYSSWFYIFPKGIRHFLNYTKDTYNDPVIYVTENGVDNYN 420
2A.52           TDSGVNATPYDLNGNLIGPQAYSSWFYIFPKGIRHFLNYTKDTYNDPVIYVTENGVDNYN 420
2A.55           TDSGVNATPYDLNGNLIGPQAYSSWFYIFPKGIRHFLNYTKDTYNDPVIYVTENGVDNYN 420
2A.62           TDSGVNATPYDLNGNLIGPQAYSSWFYIFPKGIRHFLNYTKDTYNDPVIYVTENGVDNYN 420
                ************************************************************

pCAS5           NESQPIEEALQDDFRISYYKKHMWNALGSLKNYGVKLKGYFAWSYLDNFEWNIGYTSRFG 480
2A.32           NESQPIEEALQDDFRISYYKKHMWNALGSLKNYGVKLKGYFAWSYLDNFEWNIGYTSRFG 480
2A.52           NESQPIEEALQDDFRISYYKKHMWNALGSLKNYGVKLKGYFAWSYLDNFEWNIGYTSRFG 480
2A.55           NESQPIEEALQDDFRISYYKKHMWNALGSLKNYGVKLKGYFAWSYLDNFEWNIGYTSRFG 480
2A.62           NESQPIEEALQDDFRISYYKKHIWNALGSLKNYGVKLKGYFAWSYLDNFEWNIGYTSRFG 480
                **********************:************************************

pCAS5           LYYVDYKNNLTRYPKKSAHWFTKFLNISVNANNIYELTSKDSRKVGKFYVM 531    % ID
2A.32           LYYVDYKNNLTRYPKKSAHWFTKFLNISVNANNIYELTSKDSRRVGKFYVM 531      99
2A.52           LYYVDYKNNLTRYPKKSAHWFTKFLNISVNANNIYELTSKDSRKVGKFYVM 531      99
2A.55           LYYVDYKNNLTRYPKKSAHWFTKFLNISVNANNIYELTSKDSRKVGKFYVM 531      99
2A.62           LYYVDYKNNLTRYPKKSAHWFTKFLNISVNANNIYELTSKDSRKVGKFYVM 531      98
                ******************************************:*******
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Figure 2 Multiple sequence alignment of derived amino acid sequences from four cDNA clones and pCAS5. The highly conserved TFNEP and VTENG motifs, containing the catalytic acid/base (Glu198) and catalytic nucleophile (Glu413), respectively, are underlined. The putative N-glycosylation sites are boxed. % ID indicates percent sequence identity to the amino acid sequence derived from pCAS5. *****, single, fully conserved residue; **:**, conservation of strong group; **.**, conservation of weak group; -, no consensus.

be reasonably assumed to be catalytically active in cassava. Previously, three isozymes of cassava linamarase, with p*I* values of 4.3, 3.3 and 2.9 and a single molecular weight of 63,000, were identified by chromatofocusing column chromatography (Eksittikul and Chulavatanatol, 1988). However, the chromatofocusing procedure was done in pH ranged between 5-1, which did not cover the p*I* values of the four polypeptides derived in this study and that of the polypeptide derived from pCAS5, which exhibited p*I* values between 5.3-5.6. There may be many other isoforms of linamarase, which are expressed at different times or in different parts of the plant. So, the four cDNA sequences isolated in this report (2A.32, 2A.52, 2A.55 and 2A.62) would represent only a subset of a large number of glycosidase sequences presented in the cassava genome. The two other cDNA sequences (2A.29 and 2A.33) that contained mutations and hence premature terminations could represent "pseudogenes", or "dead genes". Pseudogenes are defined as genes of similar sequences to normal genes but contain disablements, such as frameshifts or stop codons in the middle of the coding region, such that their translation would result in non-functional products. So, it is noteworthy that pseudogenes can be transcribed, but the functions of some transcripts are not known (Snyder and Gerstein, 2003).

It is not uncommon to observe in plant genomes the existence of multigene family of family 1 β-glycosidase enzymes, of which cyanogenic β-glucosidases are a member. Indeed, at least 48 sequences (including 8 probable pseudogenes) of glycosidase have been identified in the genome of *Arabidopsis thaliana*. There may be 50 members of these enzymes in the rice genome, whereas at least 16 members were recognized in the maize genome (Xu *et al*., 2004). The presence of multiple forms of enzymes may reflect functional redundancy among the gene products. On the other hand, plant may require a variety of β-glycosidase enzymes, with distinct aglycone specificities or expression patterns, for catabolism of diverse β-glycosidic metabolites for their normal growth and development (Xu *et al*., 2004).

In summary, six different cDNA clones of linamarase from cassava have been isolated by RT-PCR. Only four clones encode full-length polypeptides of linamarase. The nucleotide and amino acid sequences of these four clones revealed high sequence identity to the previously reported sequence of cassava linamarase. The full-length cDNA sequences obtained from this study will be used for further recombinant protein expression, site-directed mutagenesis and characterization in order to elucidate the structure-function relationships in this enzyme.

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ลำดับนิวคลีโอไทด์และลำดับกรดอะมิโน ของเอนไซม์ไซยาโนจีนิคเบต้ากลูโคซิเดส (ลินามาเรส) จากมันสำปะหลัง

Nucleotide and derived amino acid sequences of the cyanogenic beta-glucosidase (linamarase) from cassava (*Manihot esculenta* Crantz)

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Prachumporn Toonkool and Nusra Tongtubtim

บทคัดย่อ

มันสำปะหลังมีเอนไซม์ไซยาโนจีนิค เบต้า-กลูโคซิเดสหลายชนิด แต่ได้มีการรายงานลำดับนิวคลีโอไทด์ของ cDNA เพียงชนิดเดียวเท่านั้น (pCAS5) ในการศึกษาความสัมพันธ์ระหว่างโครงสร้างและการทำงานของเอนไซม์ไซยาโนจีนิค เบต้า-กลูโคซิเดส (ลินามาเรส) ผู้วิจัยได้ทำการโคลน cDNA ของยีนไซยาโนจีนิค เบต้า-กลูโคซิเดส จากมันสำปะหลังทั้งหมด 6 โคลน ด้วยปฏิกิริยา reverse transcription-polymerase chain reaction โดยใช้ไพรเมอร์ที่มีลำดับเบสเหมือนกับลำดับเบสของ pCAS5 พบว่าลำดับนิวคลีโอไทด์ของทั้งหมด 6 โคลนที่ได้มีความเหมือนกับลำดับนิวคลีโอไทด์ของ pCAS5 ถึง 98-99% และพบว่า cDNA 4 โคลน จาก 6 โคลน จะมีลำดับกรดอะมิโนที่เหมือนกับลำดับกรดอะมิโนที่แปลผลได้จาก pCAS5 ถึง 98-99% ส่วน cDNA อีก 2 โคลน พบว่ามีลำดับนิวคลีโอไทด์ที่ทำให้แปลรหัสได้สายโปลีเปบไทด์ที่สั้นกว่าปกติ (เนื่องจากเกิด premature termination)

Abstract

Many isozymes of cassava cyanogenic β-glucosidase (linamarase) exist, but only one cDNA sequence (pCAS5) has been reported thus far. In order to study structure-function relationships in this enzyme, the cDNA of cassava linamarase was cloned for further expression and characterization. In this report, six different cDNA sequences of cyanogenic β-glucosidase from cassava were cloned by reverse transcription-polymerase chain reaction using primers designed from the sequence of pCAS5. Nucleotide sequences of all six clones showed between 98-99% sequence identity to the nucleotide sequence of pCAS5. Derived amino acid sequences from four cDNA clones showed between 98-99% sequence identity to that of pCAS5, while the other two cDNA clones contained nucleotide sequences that led to premature termination.

Key words: beta-glucosidase, linamarase, cassava, nucleotide sequence, amino acid sequence

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Introduction

Cassava (*Manihot esculenta* Crantz) is an important crop due to the large storage roots which are a staple carbohydrate source for many populations worldwide. Young cassava leaves are also consumed by humans or used as animal feeds (Yeoh, 1989). However, both leaves and roots of cassava are cyanogenic, posing a potential health hazard to consumers. Cyanogenesis in cassava involves two cyanogenic glucosides (linamarin and lotaustralin) and sequential action of two enzymes (β-glucosidase and hydroxynitrile lyase). Normally, the glucoside substrates and the enzymes are kept in separate compartments. Exposure of these two cyanogenic glucosides to β-glucosidase, following tissue damage, results in their hydrolysis to yield glucose and an aglycone, which is acetone cyanohydrin or butanone cyanohydrin. These cyanohydrins may be broken down spontaneously or enzymatically by α-hydroxynitrile lyase to produce hydrogen cyanide (Eksittikul and Chulavatanatol, 1988 and Hughes *et al*., 1992). The cyanogenic β-glucosidase, or linamarase, which is responsible for the first step of cassava cyanogenesis, is present in many parts of the plant, including petioles, stem, leaves, peels and tuber cortex. Physiologically, the enzyme plays an important role as a host defense against biological predators. Furthermore, an *in vitro* study has discovered the unique ability of cassava β-glucosidase in alkyl glucoside synthesis transferring glucose to a large variety of alcohol acceptors, while β-glucosidases from Thai rosewood and almond could not (Svasti *et al*., 2003). This difference in enzymatic action may lie in slight variations in the amino acid sequences of β-glucosidases from various sources. So, it is of scientific interest to understand the molecular basis of enzymatic catalysis in cassava β-glucosidase. To achieve this, cassava β-glucosidase must be cloned and expressed as a recombinant protein, its nucleotide sequence mutated, the effects of mutation characterized. These procedures will help to identify amino acid residues responsible for transglucosylation activity.

A number of studies have shown the existence of multiple forms of cassava β-glucosidase (Eksittikul and Chulavatanatol, 1988 and Yeoh, 1989), however only a single cDNA clone of cyanogenic β-glucosidase (pCAS5) from cassava has been isolated thus far (Hughes *et al*., 1992). The identity of other cassava β-glucosidase isozymes has not been reported. In this study, six different cDNA clones of cyanogenic β-glucosidase from cassava have been isolated by RT-PCR. Only four clones encode full-length polypeptides of cyanogenic β-glucosidase. The nucleotide and amino acid sequences of these four clones revealed high sequence identity to the previously reported sequence of cassava β-glucosidase. The full-length cDNA sequences obtained from this study will be used for further recombinant protein expression, site-directed mutagenesis and characterization in order to elucidate the structure-function relationship in this enzyme.

Materials and Methods

Total RNA was isolated from young cassava leaves (local varieties) using RNeasy extraction kit (QIAGEN, USA) and concentration estimated by absorbance at 260 nm. 0.2-5 μg total RNA was converted into single stranded first strand cDNA fragment using 200 U SuperScript II reverse transcriptase (Invitrogen, USA) in the reaction containing 0.5 μg oligo$(dT)_{12-18}$ primer, 10 nmol dNTP mix, and 10 mM DTT at 42 °C for 50 min and at 70 °C for 15 min. The RNA strand of the RNA:cDNA duplex was degraded by 2 U RNase H at 37 °C for 20 min. 1 μL aliquot of the product was used as a template for PCR with 1 μM each of CVLMF2 (5' TTC TTC AGC TAT CAG GGA TGC 3') and CVLMR2 (5' TGC TAG ATC ATT GGA GCT TCA 3') primers, 1.5 mM $MgCl_2$, 50 mM KCl, 0.1% Triton X-100, 0.2 mM of each dNTP and 2.5 U Expand HiFi DNA polymerase (Boehringer, Germany) in a 50-μL reaction. All PCR reactions were done using a 94 °C initial denaturation for 2 min, 29 cycles of 94 °C for 60 s, 45 °C for 30 s, and 72 °C for 2 min, and a 72 °C final elongation for 8.5 min. The primer sequences correspond to bases 7-27 (sense) and bases 1677-1697 (antisense), respectively, of the cassava cyanogenic β-glucosidase cDNA clone pCAS5 reported previously (Hughes, *et al*., 1992). The resulting RT-PCR product was gel-purified, ligated into pGEM-Teasy vector (Promega, USA) and transformed into *E. coli* DH5α.

The nucleotide sequence of cDNA clones were determined by automated DNA sequencing (ABI 373A DNA sequencer). The nucleotide sequences obtained were translated into amino acid sequences using EMBOSS Transeq program. Alignment of nucleotide and amino acid sequences were done using ClustalW version 1.82. The physical and chemical parameters of the derived amino acid sequences were performed using ProtParam program.

Results and Discussion

Cloning of cDNA of cassava cyanogenic β-glucosidase

Total RNA (48 ng/μL) was extracted from young cassava leaves. Approximately 500 ng total RNA was used to synthesized first strand cDNA. PCR amplification of first strand cDNA yielded 1.7 kb PCR product (Fig. 1), which was then ligated into pGEM-Teasy vector and transformed into *E. coli* DH5α. The nucleotide sequences of cloned RT-PCR products were determined by automatic sequencing, yielding six different cDNA clones, namely 2A.29, 2A.32, 2A.33, 2A.52, 2A.55 and 2A.62. Alignment of the nucleotide sequences from all six cDNA clones shows between 98-99% sequence identity to the previously reported nucleotide sequence of the cassava cyanogenic β-glucosidase cDNA clone pCAS5 (Hughes, *et al*., 1992), and to each other (results not shown).

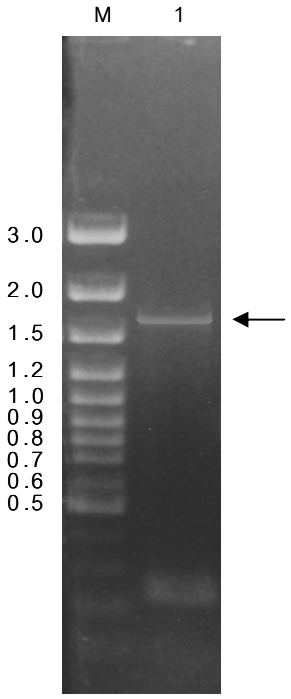

Fig. 1: cDNA of cyanogenic β-glucosidase from cassava (analyzed on 1% (w/v) agarose and stained with ethidium bromide). Lane 1, the 1.7 kb RT-PCR product (indicated by an arrow); M, DNA size marker.

Alignment of nucleotide and derived amino acid sequences of cassava cyanogenic β-glucosidase

The nucleotide sequences from 6 cDNA clones were translated and aligned. A thymine deletion in clone 2A.29, corresponding to position 570 in pCAS5, results in a frame-shift mutation from that position onward and a pre-mature termination 7 amino acids later. Also in clone 2A.33, an adenine-to-thymine substitution, corresponding to position 225 in pCAS5, results in a non-sense mutation at that position. So, only four cDNA clones, namely 2A.32, 2A.52, 2A.55 and 2A.62, encode the full-length amino acid sequence of cassava cyanogenic β-glucosidase. Alignment of the amino acid sequences derived from these four cDNA clones shows between 98-99% sequence identity to the previously reported amino acid sequence of the cassava cyanogenic β-glucosidase derived from pCAS5 (Hughes, *et al*., 1992), and to each other (Fig. 2). All four predicted polypeptides contain the highly conserved TFNEP and VTENG motifs bearing the catalytic acid/base (Glu198) and catalytic nucleophile (Glu413), respectively. The five putative N-glycosylation motifs, NX(S/T), are also conserved. The polypeptides derived from cDNA clones were analyzed for their physical and chemical parameters (Table 1).

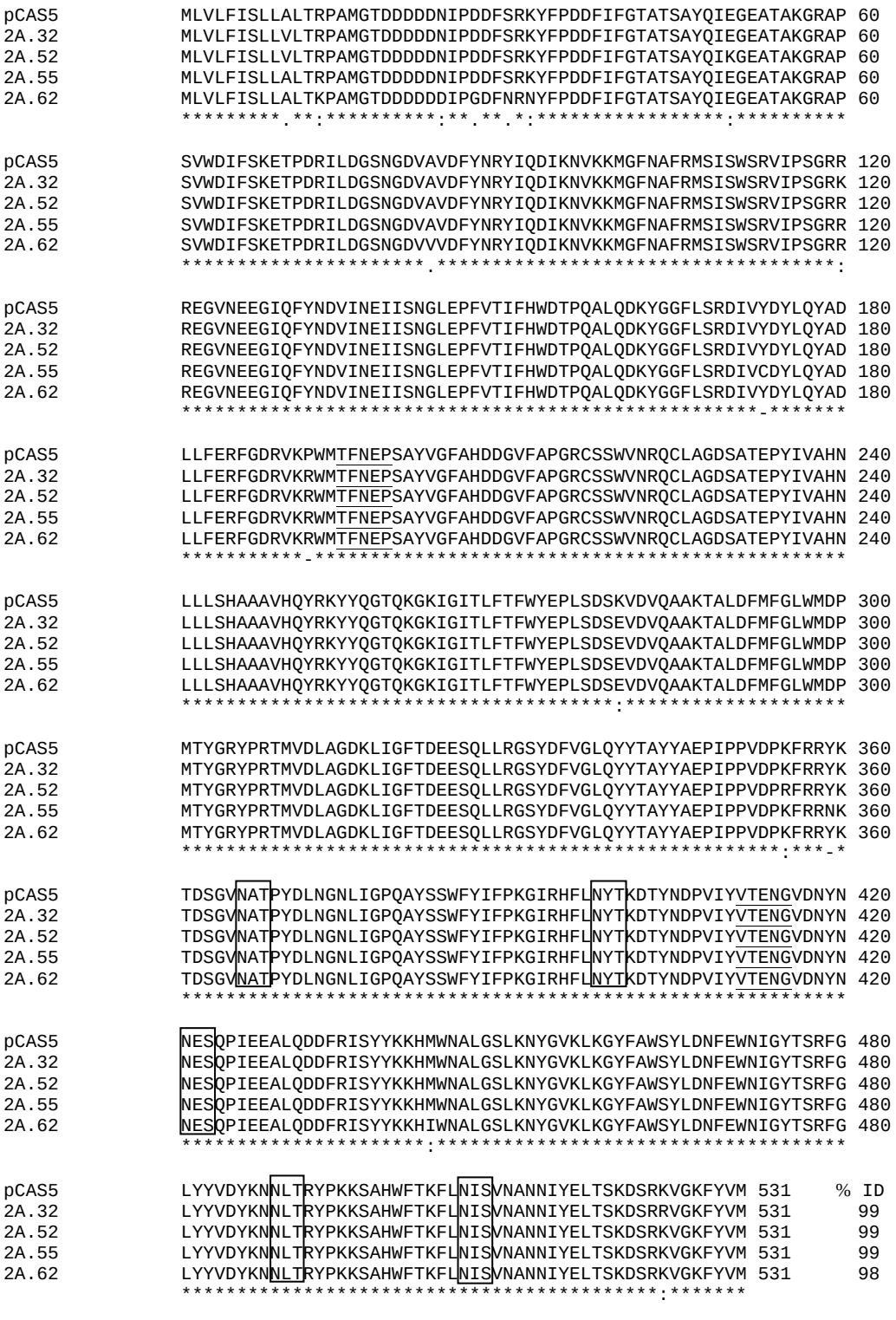

Fig. 2: Multiple sequence alignment of derived amino acid sequences from four cDNA clones and pCAS5. The highly conserved TFNEP and VTENG motifs, containing the catalytic acid/base (Glu198) and catalytic nucleophile (Glu413), respectively, are underlined. The putative N-glycosylation sites are boxed. % ID indicates percent sequence identity to the amino acid sequence derived from pCAS5. *****, single, fully conserved residue; :, conservation of strong group; ., conservation of weak group; -, no consensus.

Table 1: Physical and chemical parameters of the polypeptides derived from four cDNA clones.

Name	Number of amino acids	Molecular weight	Theoretical p*I*
pCAS5	531	61,372.9	5.52
2A.32	531	61,461.0	5.44
2A.52	531	61,488.1	5.62
2A.55	531	61,323.8	5.44
2A.62	531	61,370.8	5.35

The cDNA sequences reported here were synthesized from young cassava leaves, which had been shown to be the site of cassava cyanogenic β-glucosidase synthesis (Poncoro and Hughes, 1992). This is likely to produce different cyanogenic β-glucosidase transcripts from the previously reported cassava cyanogenic β-glucosidase cDNA sequence, pCAS5, which was extracted from yellow cotyledons (Hughes *et al*., 1992). The four polypeptides predicted from the four cDNA clones are very similar to the previously reported cassava cyanogenic β-glucosidase, in terms of sequence identity, physico-chemical properties as well as the presence of the key motifs essential for catalytic activity. So, these four predicted polypeptides can be reasonably assumed to be catalytically active in cassava. Previously, three isozymes of cyanogenic β-glucosidase, with p*I* values of 4.3, 3.3 and 2.9 and a single molecular weight of 63,000, were identified by chromatofocusing column chromatography (Eksittikul and Chulavatanatol, 1988). However, the chromatofocusing procedure was done in pH ranges between 5-1, which did not cover the p*I* values of the four polypeptides derived in this study, as well as the previously reported sequence of cassava cyanogenic β-glucosidase (all exhibiting p*I* values between 5.3-5.6). There may be many other isoforms of cyanogenic β-glucosidase, which are expressed at different times or in different parts of the plant. So, the four cDNA sequences isolated in this report (2A.32, 2A.52, 2A.55 and 2A.62) would represent only a subset of a large number of glycosidase sequences present in the cassava genome. The two cDNA sequences (2A.29 and 2A.33) that contain mutations and hence premature terminations could represent “pseudogenes”, or “dead genes”. Pseudogenes are defined as genes of similar sequences to normal genes but containing disablements, such as frameshifts or stop codons in the middle of the coding region, such that their translation would result in non-functional products. So, it is noteworthy that pseudogenes can be transcribed, and functions of some transcripts are not known (Snyder and Gerstein, 2003).

It is not uncommon to observe in plant genomes the existence of multigene family of family 1 β-glycosidase enzymes (of which cyanogenic β-glucosidases are a member). Indeed, at least 48

sequences (including 8 probable pseudogenes) of glycosidase have been identified in the genome of *Arabidopsis thaliana*. There may be 50 members of these enzymes in the rice genome, whereas at least 16 members were recognized in the maize genome (Xu *et al*., 2004). The presence of multiple forms of enzymes may reflect functional redundancy among the gene products. On the other hand, plant may require a variety of β-glycosidase enzymes, with distinct aglycone specificities or expression patterns, for catabolism of diverse β-glycosidic metabolites for their normal growth and development (Xu *et al*., 2004).

In summary, four full-length cDNA sequences of cassava cyanogenic β-glucosidase have been cloned. These cDNA clones will be subcloned into expression vectors, expressed as recombinant proteins and their functions tested. Active recombinant enzymes may be used for future protein engineering studies.

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การกลายพันธุ์ที่ตำแหน่งจำเพาะในช่องจับอะไกลโคนของเอนไซม์เบต้า-กลูโคซิเดสจากพะยูง (*Dalbergia cochinchinensis* Pierre)

Site-directed Mutagenesis in the Aglycone Binding Pocket of Beta-Glucosidase from Thai Rosewood (*Dalbergia cochinchinensis* Pierre)

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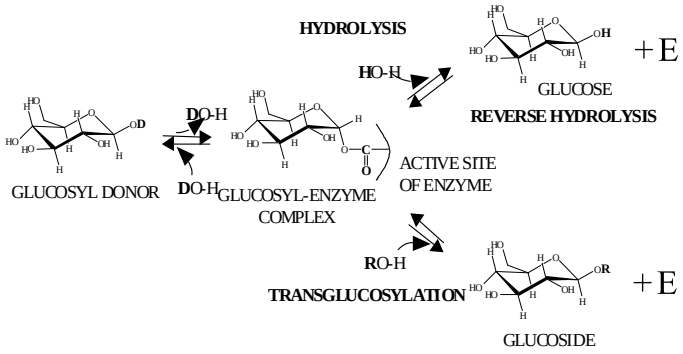
บทคัดย่อ: เอนไซม์เบต้า-กลูโคซิเดสไม่เพียงแต่สลายพันธะไกลโคซิดิคเท่านั้น เอนไซม์บางชนิดยังสามารถเร่งปฏิกิริยาย้อนการสลายและปฏิกิริยาการย้ายหมู่กลูโคสได้อีกด้วย ดัลโคชิเนสซึ่งเป็นเอนไซม์เบต้า-กลูโคซิเดสจากพะยูงทำหน้าดีกว่าในปฏิกิริยาย้อนการสลาย แต่แย่กว่าในปฏิกิริยาการย้ายหมู่กลูโคส เมื่อเปรียบเทียบกับลินามาเรส ซึ่งเป็นเอนไซม์เบต้า-กลูโคซิเดสจากมันสำปะหลัง เอนไซม์ทั้งสองต่างมีความจำเพาะกับสับสเตรทธรรมชาติของมัน (ดัลโคชินินกลูโคไซด์และลินามาริน ตามลำดับ) ดังนั้นเอนไซม์ทั้งสองจึงมีความแตกต่างทั้งในการเร่งปฏิกิริยาและความจำเพาะต่อสับสเตรท ทั้งๆที่มีลำดับกรดอะมิโนคล้ายกันถึง 70% งานวิจัยนี้มีวัตถุประสงค์เพื่อหากรดอะมิโนที่มีความสำคัญต่อการทำงานของดัลโคชิเนส โดยการแทนที่กรดอะมิโนในช่องจับอะไกลโคนของดัลโคชิเนสด้วยกรดอะมิโนของลินามาเรส (H253F และ N323Q) ดัลโคชิเนสกลายพันธุ์จะถูกแสดงออกโดยยีสต์ *Pichia pastoris* และทำให้บริสุทธิ์ ดัลโคชิเนสกลายพันธุ์ทั้งสองชนิดมีลักษณะคล้ายกับดัลโคชิเนสก่อนกลายพันธุ์ เมื่อพิจารณาจาก SDS-PAGE, western blot และ การย้อมกิจกรรมเอนไซม์บน non-denaturing PAGE จากการเปรียบเทียบกิจกรรมของดัลโคชิเนสกลายพันธุ์และดัลโคชิเนสธรรมชาติ ในการสลายสับสเตรทชนิดต่างๆ พบว่ามีค่าลดลง แสดงว่าทั้ง H253 และ N323 อาจมีความสำคัญในการกำหนดความจำเพาะต่อสับสเตรท และการเร่งปฏิกิริยาของเอนไซม์ดัลโคชิเนส

Abstract: β-Glucosidases not only hydrolyze of β-glucosidic linkage, but some also catalyze reverse hydrolysis and transglucosylation. Dalcochinase (Thai rosewood β-glucosidase) was better for reverse hydrolysis, but poorer for transglucosylation, when compared with linamarase (cassava β-glucosidase). Both enzymes also exhibit specificities for their natural substrates (dalcochinin glucoside and linamarin, respectively). So they show differences in catalytic properties and substrate preferences, despite sharing 70% amino acid sequence identity. This research aims to identify key amino acid residues for function of dalcochinase by replacing the residues in the aglycone binding pocket of dalcochinase with the corresponding residues of linamarase (H253F and N323Q). Mutant enzymes were expressed by *Pichia pastoris* and purified. Both mutants appeared similar to wild-type dalcochinase as judged by SDS-PAGE, western blot and activity staining on non-denaturing PAGE. The decrease in relative hydrolytic activities of both mutants towards various substrates, compared with natural dalcochinase, suggested that both H253 and N323 may play important roles in determining substrate specificity and catalytic functions of dalcochinase.

Introduction: β-Glucosidases catalyze the hydrolysis of β-glucosidic linkage between glycone (glucose) and aglycone group or other sugars. Furthermore, some of them exhibit additional capabilities in catalyzing reverse hydrolysis and transglucosylation reactions (Scheme 1). Different enzymes show a variety of natural substrate specificities. For example, Thai rosewood β-glucosidase, or dalcochinase, hydrolyzes dalcochinin glucoside, which is its natural substrate, but does not hydrolyze linamarin. In contrast, cassava β-glucosidase, or linamarase, hydrolyzes linamarin, which is its natural substrate, but does not hydrolyze dalcochinin glucoside. In terms of catalytic activities, dalcochinase catalyzes reverse hydrolysis well, but acts moderately in transglucosylation. In contrast, linamarase is poor in catalyzing reverse hydrolysis, but highly efficient in catalyzing transglucosylation. So these two enzymes show differences in catalytic properties and diverse substrate preferences even though they show 70% amimo acid sequence identity. Therefore, we are interested in identifying the specific amino acid residues that play important roles in determining substrate specificity and catalytic functions of dalcochinase by using site-directed mutagenesis.



Scheme 1: Hydrolysis, reverse hydrolysis and transglucosylation reactions of β-glucosidases

Methodology: The three-dimensional structure of *Trifolium repens* β-glucosidase (1CBG) and the alignment of 1CBG, dalcochinase and linamarase were used to determine the amino acid residues that are different between dalcochinase and linamarase, and are likely located in the algycone binding pocket. Site-directed mutagenesis was used to replace the amino acid residues of dalcochinase with the corresponding amino acid residues of linamarase, generating H253F and N323Q dalcochinase mutants. The mutant enzymes were expressed in *Pichia pastiris*, which were grown in minimal media plus 1% glycerol overnight at 30 °C, 200 rpm, and inoculated into minimal media plus 0.5% methanol and 0.5% casamino acid for 15 days for expression. The mutant enzymes were purified from culture media via phenyl sepharose chromatography, immobilized metal affinity chromatography (IMAC) and ultrafiltration centrifuge, respectively. The purified enzymes were characterized by SDS-PAGE and detected by monoclonal antibody against natural dalcochinase. Activities of purified enzymes on non-denaturing PAGE were detected by 1 mM 4-methyllumbelliferyl-β-D-glucoside (4-MU-Glc). The activities of mutant and wild-type enzymes towards *p*-nitrophenyl glucoside (*p*NP-Glc), linamarin and dalcochinin glucoside were determined by incubating 20 mU enzyme with 2 mM substrate in 0.1 M sodium acetate, pH 5.0, at 30 °C for 5 min, and stopping the reaction by boiling for 2 min. The liberated glucose was measured by using glucose oxidase reagent and reading absorbance at 505 nm.

Results, Discussion, and Conclusion: H253 and N323 of dalcochinase were chosen for site-directed mutagenesis because their corresponding positions (V254 and N324,

respectively) are located in the aglycone binding pocket of 1CBG. Site-directed mutagenesis was used to replace H253 and N323 of dalcochinase into F and Q, which are the corresponding residues in linamarase (Fig. 1), thereby generating H253F and N323Q mutants, respectively. The mutant enzymes were cloned, expressed in *P. pastoris* and purified as describe above. The purified mutant enzymes showed a broad protein band around 66 kD by SDS-PAGE, which were detected by monoclonal antibody against natural dalcochinase (Fig. 2, a and b). Both mutants were active when staining with 4-MU-Glc on non-denaturing PAGE (Fig. 3, a and b). The relative hydrolytic activities towards *p*NP-Glc, linamarin and dalcochinin glucoside of both mutant enzymes decreased when compared with natural dalcochinase (Table 1), suggesting that both H253 and N323 may play important roles in determining substrate specificity and catalytic functions of dalcochinase. Also, both mutants showed lower hydrolytic activities towards linamarin than linamarase, suggesting that F and Q were not involved in substrate specificity and catalytic functions of linamarase.

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                      240       250       260       270       280       290
1CBG             238 KTKYQASQNGIIGITLVSHWFEPASKEKADVDAAKRGLDFMLGWFMHPLTKGRYPESMRY 297
TRglu            237 KRKYQEHQKGTIGISLHVVWVIPLSNSTSDQNATQRYLDFTCGWFMDPLTAGRYPDSMQY 296
CVLM             253 RKYYQGTQKGKIGITLFTFWYEPLSDSKVDVQAAKTALDFMFGLWMDPMTYGRYPRTMVD 312

                      300       310       320       330       340       350
1CBG             298 LVRKRLPKFSTEESKELTGSFDFLGLNYYSSYYAAKAPRIPNARPAIQTDSLINAT-FE- 355
TRglu            297 LVGDRLPKFTTDQAKLVKGSFDFIGLNYYTTNYATKSDASTCCPPSYLTDPQVTLL-QQ- 354
CVLM             313 LAGDKLIGFTDEESQLLRGSYDFVGLQYYTAYYAEPIPPVDPKFRRYKTDSGVNATPYD- 371
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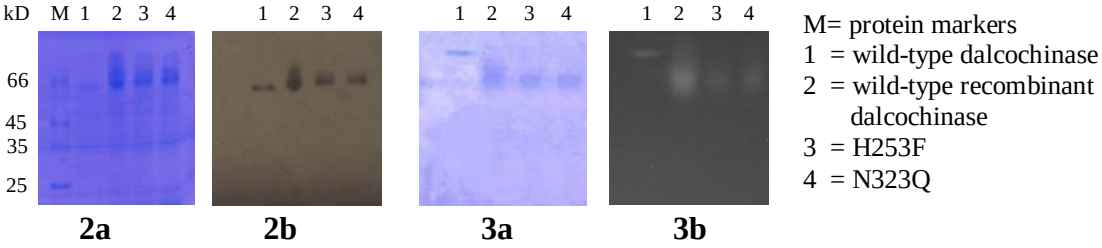
Fig. 1: The alignment of 1CBG, dalcochinase (TRglu) and linamarase (CVLM). The shadow indicate the target sites for site-directed mutagenesis.



2a **2b** **3a** **3b**

Fig. 2: SDS-PAGE of purified enzymes. (a) Coomasie stain; (b) Western blot.
Fig. 3: Non-denaturing PAGE of purified enzymes. (a) Coomasie stain; (b) staining with 1 mM 4-MU-Glc.

Table 1: Relative hydrolytic activities of dalcochinase, linamarase, and H253F and N323Q mutants, with respect to hydrolytic activity of dalcochinase towards pNP-Glc.

Substrates (2 mM)	Dalcochinase	Linamarase	H253F	N323Q
pNP-Glc	100.00	74.11	84.04	34.22
Linamarin	2.130	21.10	0.35	1.06
Dalcochinin glucoside	124.65	1.42	97.34	58.33

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Keywords:β-glucosidase, *Dalbergia cochinchinensis* Pierre, site-directed mutagenesis, substrate specificity ,Thai rosewood, transglucosylation

การผลิตและการศึกษาคุณลักษณะของเอนไซม์เบต้า-กลูโคซิเดสกลายพันธุ์จากพะยูง

PRODUCTION AND CHARACTERIZATION OF MUTANT FORMS OF THAI ROSEWOOD β-GLUCOSIDASE

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บทคัดย่อ: เอนไซม์เบต้า-กลูโคซิเดสจากสิ่งมีชีวิตชนิดต่างๆมีความจำเพาะต่อสับสเตรทที่ต่างกัน ดัลโคชิเนสซึ่งเป็นเอนไซม์เบต้า-กลูโคซิเดสจากพะยูง มีความจำเพาะต่อดัลโคชินิน เบต้า-กลูโคไซด์ ขณะที่ลินามาเรสซึ่งเป็นเอนไซม์เบต้า-กลูโคซิเดสจากมันสำปะหลัง มีความจำเพาะต่อลินามาริน ดัลโคชิเนสสามารถเร่งปฏิกิริยาย้อนการสลายได้ดีแต่เร่งปฏิกิริยาการย้ายหมู่กลูโคสได้ปานกลาง ในทางตรงข้ามลินามาเรสสามารถเร่งปฏิกิริยาการย้ายหมู่กลูโคสได้ดีมากแต่เร่งปฏิกิริยาย้อนการสลายได้น้อย งานวิจัยนี้จะทำการกลายพันธุ์ดัลโคชิเนส 2 ตำแหน่ง คือ I185A และ E455I โดยการแทนที่กรดอะมิโนในบริเวณที่คาดว่าทำหน้าที่จับกับหมู่อะไกลโคนของดัลโคชิเนสด้วยกรดอะมิโนของลินามาเรส ทำการโคลนดัลโคชิเนสกลายพันธุ์และให้มีการแสดงออกในยีสต์ *Pichia pastoris* แล้วสกัดให้บริสุทธิ์จากอาหารเลี้ยงเชื้อ ดัลโคชิเนสกลายพันธุ์ทั้ง 2 ตัว มีลักษณะคล้ายกับดัลโคชิเนสก่อนกลายพันธุ์ เมื่อวิเคราะห์โดย SDS-PAGE, western blot และ ย้อมกิจกรรมเอนไซม์บน non-denaturing PAGE ผลจากการเปรียบเทียบกิจกรรมการสลายสับสเตรทชนิดต่างๆ ของดัลโคชิเนสกลายพันธุ์กับดัลโคชิเนสก่อนกลายพันธุ์และลินามาเรส ชี้ให้เห็นว่า เฉพาะ I185 เพียงตัวเดียวเท่านั้นที่น่าจะมีความสำคัญในการจับกับ ดัลโคชินิน เบต้า-กลูโคไซด์ และ *p*-nitrophenyl β-glucoside

Abstract: *β*-Glucosidases from different organisms exhibit diverse substrate specificities. Dalcochinase (a *β*-glucosidase from Thai rosewood) shows specificity for dalcochinin *β*-glucoside, while linamarase (a *β*-glucosidase from cassava) has specificity for linamarin. Dalcochinase is capable of catalyzing reverse hydrolysis, but modest in catalyzing transglucosylation. In contrast, linamarase is excellent in catalyzing transglucosylation, but poor in catalyzing reverse hydrolysis. In this project, we constructed 2 dalcochinase mutants, namely I185A and E455I, by replacing amino acid residues that are likely located in the aglycone binding site of dalcochinase with the corresponding residues of linamarase. Mutant enzymes were cloned and expressed in yeast *Pichia pastoris*, and purified from culture media. Both mutants are similar to wild-type dalcochinase as shown by SDS-PAGE, western blot and activity staining on non-denaturing PAGE. Comparison of hydrolytic activities of mutant and wild-type enzymes towards various substrates suggested that only I185 may be important for binding to dalcochinin *β*-glucoside and *p*-nitrophenyl β-glucoside.

Introduction: *β*-Glucosidases (EC 3.2.1.21) are present in a wide range of organisms. They catalyze the hydrolysis of *β-O*-glucosidic linkage between D-glucose and

aglycone or another sugar. Apart from hydrolysis, *β*-glucosidases can catalyze reverse hydrolysis, giving glucose disaccharides and trisaccharides as products when using free glucose as substrate. They also catalyze transglucosylation reaction by transferring glucose from donor (*p*-nitrophenyl β-glucoside, pNP-Glc) to acceptor (alcohol) and giving alkyl glucoside as product. All *β*-glucosidases are similar in specificity for the glycone moiety, but some enzymes, especially plants' enzymes, show variable specificities for different aglycone moieties. For example, dalcochinase from Thai rosewood (*Dalbergia cochinchinensis* Pierre) is specific for dalcochinin *β*-glucoside, while linamarase from cassava (*Manihot esculenta* Crantz) is specific for linamarin. In addition, dalcochinase has high efficiency in catalyzing reverse hydrolysis, but linamarase has high efficiency in catalyzing transglucosylation. As a result of the differences in substrate specificities and abilities in catalyzing reverse hydrolysis and transglucosylation reactions, we are interested in delineating the structure-function relationships in plants *β*-glucosidases by using dalcochinase and linamarase as models.

Methodology: From the sequence alignment of *Trifolium repens β*-glucosidase (1CBG), dalcochinase and linamarase, and from the three-dimensional structure of 1CBG, the different residues between dalcochinase and linamarase that are likely located in the aglycone binding site were identified. Mutant enzymes, namely I185A and E455I, were generated by replacing amino acid residues of dalcochinase with the corresponding residues of linamarase by using QuickChang®II Site-Directed Mutagenesis Kit. Mutant enzymes were cloned in *Escherichia coli*, sequenced, and expressed in yeast *Pichia pastoris* under methanol induction. The cultures were grown in minimal media containing 1% glycerol overnight at 30 °C, 200 rpm. Then, they were inoculated into minimal media containing 0.5% methanol and 0.5% casamino acid and grown for 12 days for expression. The mutant enzymes were secreted into culture medium, and purified via phenyl sepharose column and Ni^{2+} ion affinity chromatography, respectively. Purified mutant enzymes were characterized by SDS-PAGE, western blot using monoclonal antibody against natural dalcochinase, as well as non-denaturing PAGE and staining with 1 mM 4-methylumbelliferyl-*β-D*-glucoside (4-MU-Glc). The hydrolytic activities of mutant enzymes and wild-type dalcochinase and linamarase were studied by incubating 0.02 U enzyme with 2 mM substrate (dalcochinin *β*-glucoside, linamarin and pNP-Glc) in 0.1 M sodium acetate, pH 5.0, in 0.1 ml reaction at 30 °C for 5 min. The reactions were stopped by boiling for 2 min. The glucose released was detected by adding 1 ml glucose oxidase reagent and measuring absorbance at 505 nm.

Results, Discussion, and Conclusion: The alignment of 1CBG, dalcochinase and linamarase identified several residues that are different between dalcochinase and linamarase (Fig. 1). Of these, we selected I185 and E455 in dalcochinase as our target sites for site-directed mutagenesis because their corresponding residues in 1CBG (G186 and S466) are located in the aglycone binding pocket in the structure of 1CBG. I185 and E455 were mutated into A and I, which were the corresponding residues in linamarase, generating I185A and E455I mutants, respectively. These mutants were cloned, expressed and purified as described. SDS-PAGE showed both mutants as a broad band with apparent molecular weight of 66-69 kD, which were detected by antibody against natural dalcochinase (Fig. 2, a and b). Both mutants showed activity towards 4-MU-Glc on non-denaturing PAGE (Fig. 3, a and b). Thus, these mutants are similar to wild-type dalcochinase. The hydrolytic activities of mutant enzymes towards

various substrates were compared with wild-type dalcochinase and linamarase (Table 1). I185A had lower ability for hydrolysis of dalcochinin *β*-glucoside and pNP-Glc than wild-type dalcochinase, but weakly hydrolyzed linamarin. E455I showed comparable activity towards dalcochinin *β*-glucoside and pNP-Glc to wild-type dalcochinase, but little activity towards linamarin. Thus, I185, but not E455, may be important for binding to dalcochinin *β*-glucoside and pNP-Glc.

```
                182      190        200        210        220        230
1CBG   178WITLNEPWGVSMNAYAYGTFAPGRCSDWLKLNCTGGDSGREPYLAAHYQLLAHAAAARLY 237
TRglu  177WITLNEPSIFTANGYAYGMFAPGRCSPSYNPTCTGGDAGTETYLVAHNLILSHAATVQVY 236
CVLM   193WMTFNEPSAYVGFAHDDGVFAPGRCSSWVNRQCLAGDSATEPYIVAHNLLLSHAAAVHQY 252

                420        430          440        450        460
1CBG   414LLDTPRIDYYYRHLYYVLTAIG-DGVNVKGYFAWSLFDNMEWDSGYTVRFGLVFVDFKNN 472
TRglu  413LIDTYRIDSYYRHLFYVRYAIR-SGANVKGFFAWSLLDNFEWAEGYTSRFGLYFVNYTT- 470
CVLM   430LQDDFRISYYKKHMWNALGSLKNYGVKLKGYFAWSYLDNFEWNIGYTSRFGLYYVDYKNN 489
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Fig. 1 Sequence alignment of 1CBG, dalcochinase (TRglu) and linamarase (CVLM).

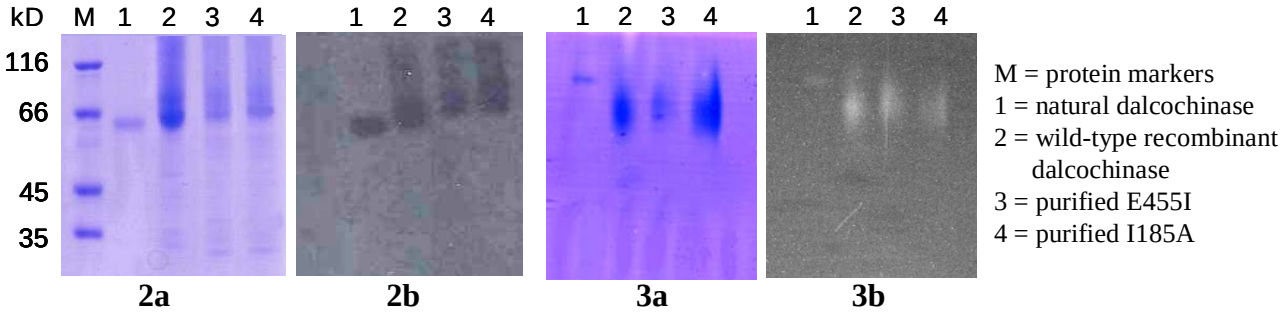

Fig. 2. SDS-PAGE of purified mutant enzymes. (a) Coomasie stain; (b) Western blot.

Fig. 3. Non-denaturing PAGE of purified mutant enzymes. (a) Coomasie stain; (b) staining with 4-MU-Glc.

Table 1. Hydrolytic activities of different β-glucosidases towards various substrates relative to activity of natural dalcochinase towards pNP-Glc.

Substrate (2 mM)	Natural dalcochinase	Linamarase	W/T recombinant dalcochinase	I185A	E455I
Dalcochinin β-glucoside	124.65	1.42	129.79	64.72	123.05
Linamarin	2.13	21.10	3.19	3.19	4.08
pNP-Glc	100.00	74.11	113.83	86.88	110.11

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Keywords: *β*-glucosidase, Thai rosewood, substrate specificity, transglucosylation, Site-directed mutagenesis

ผลจากการเกิดการกลายพันธุ์ในบริเวณจับอะไกลโคนของเอนไซม์เบต้า-กลูโคซิเดสจากพะยูง

EFFECTS OF MUTATION IN THE AGLYCONE-BINDING POCKET OF THAI ROSEWOOD β-GLUCOSIDASE.

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บทคัดย่อ: ดัลโคชิเนสเป็นเอนไซม์เบต้า-กลูโคซิเดสจากพะยูง สามารถสลาย dalcochinin β-glucoside ซึ่งเป็นสับสเตรทธรรมชาติได้ ขณะที่ลินามาเรสเป็นเอนไซม์เบต้า-กลูโคซิเดสจากมันสำปะหลัง จะสลายลินามารินได้ ดัลโคชิเนสสามารถเร่งปฏิกิริยาย้อนการสลายได้ดี แต่เร่งปฏิกิริยาย้ายหมู่กลูโคสได้น้อย ในขณะที่ลินามาเรสเร่งปฏิกิริยาย้ายหมู่กลูโคสได้ดี แต่ไม่มีประสิทธิภาพในการเร่งปฏิกิริยาย้อนการสลาย ทั้งๆที่เอนไซม์ทั้งสองมีลำดับกรดอะมิโนคล้ายคลึงกัน 60% ดังนั้นงานวิจัยนี้จึงสนใจศึกษาความสัมพันธ์ระหว่างโครงสร้างและหน้าที่ของเอนไซม์เบต้า-กลูโคซิเดส โดยหาตำแหน่งกรดอะมิโนที่สำคัญสำหรับเร่งปฏิกิริยาการสลายสับสเตรทและปฏิกิริยาย้ายหมู่กลูโคส งานวิจัยนี้ได้ทำการโคลนยีนของเอนไซม์ดัลโคชิเนส ให้มีการแสดงออกในยีสต์ *Pichia pastoris* และทำเอนไซม์ให้บริสุทธิ์ เมื่อศึกษาค่าจลนพลศาสตร์ของรีคอมบิแนนต์ดัลโคชิเนส พบว่ามีค่าคล้ายคลึงกับดัลโคชิเนสธรรมชาติ จากนั้นทำการกลายพันธุ์ที่ตำแหน่งจำเพาะของยีนดัลโคชิเนส โดยการแทนที่กรดอะมิโนในบริเวณจับอะไกลโคนของดัลโคชิเนสด้วยกรดอะมิโนที่ตำแหน่งที่ตรงกันของลินามาเรส นั่นคือเอนไซม์กลายพันธุ์ N189F และ A454N จากการศึกษาจลนพลศาสตร์ของเอนไซม์กลายพันธุ์ทั้งสองชนิด พบว่าตำแหน่ง N189 และ A454 ของเอนไซม์ดัลโคชิเนสไม่น่าจะมีส่วนเกี่ยวข้องกับการย่อยสลายลินามาริน แต่ตำแหน่ง N189 น่าจะเป็นตำแหน่งที่สำคัญต่อการย่อยสลาย *p*ara-nitrophenyl-β-D-glucoside และ dalcochinin β-glucoside เมื่อศึกษาการเร่งปฏิกิริยาย้ายหมู่กลูโคสพบว่า เอนไซม์กลายพันธุ์ N189F มีประสิทธิภาพดีขึ้น ในการเร่งปฏิกิริยาย้ายหมู่กลูโคสกับแอลกอฮอล์ชนิดปฐมภูมิ แต่ตำแหน่ง N189 และ A454 ของเอนไซม์ดัลโคชิเนสไม่น่าจะมีส่วนเกี่ยวข้องกับการเร่งปฏิกิริยาย้ายหมู่กลูโคสกับแอลกอฮอล์ชนิดทุติยภูมิและตติยภูมิ

Abstract: Dalcochinase, a β-glucosidase from Thai rosewood, can hydrolyze dalcochinin β-glucoside that is its natural substrate, whereas linamarase, a β-glucosidase from cassava, hydrolyzes its natural substrate linamarin. Dalcochinase can catalyze reverse hydrolysis well, but shows low efficiency in transglucosylation. On the other hand, linamarase catalyses transglucosylation better than dalcochinase, but was not efficient in catalyzing reverse hydrolysis. Despite these differences, both enzymes have 60% amino acid sequence homology. Thus, this project is interested in studying the relationship between structure and function of β-glucosidase, particularly the identification of the amino acid residue that is important for hydrolysis and transglucosylation. The coding sequence of dalcochinase was cloned, expressed in yeast

Pichia pastoris, and purified. The recombinant enzyme exhibits similar enzymatic properties to natural dalcochinase. Mutant forms of dalcochinase (namely N189F and A454N) were generated by replacing amino acid residues located in the aglycone binding pocket of dalcochinase with the corresponding residues of linamarase. Kinetic analysis of both enzymes showed that both N189 and A454 were not involved in hydrolysis of linamarin, but N189 could be important for hydrolysis of *para*-nitrophenyl-β-D-glucoside and dalcochinin β-glucoside. In transglucosylation studies, N189F mutant could improve transglucosylation efficiency using primary alcohols as acceptors. However, neither N189 nor A454 was likely to be involved in transglucosylation using secondary and tertiary alcohols as acceptors.

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(6) Jisnuson Svasti et al. (2003) *Biochemical and Biophysical Research Communications*, **305**, 470-475.

Keywords: β-glucosidase, Site-directed mutagenesis, Structure-function relationship, Dalcochinase, Transglucosylation

ชื่อเรื่อง: **การผลิตและการศึกษาคุณลักษณะของเอนไซม์เบต้า-กลูโคซิเดสกลายพันธุ์จากพะยูง**

ผู้แต่ง: นางสาวชมพูนุท พรเจริญนพ

สังกัด: บัณฑิตมหาวิทยาลัย มหาวิทยาลัยเกษตรศาสตร์

บทคัดย่อ: เอนไซม์เบต้า-กลูโคซิเดสจากสิ่งมีชีวิตชนิดต่างๆ จะมีความจำเพาะต่อสับสเตรทที่แตกต่างกัน โดยเฉพาะในส่วนของหมู่อะไกลโคน เอนไซม์ดัลโคชิเนสซึ่งเป็นเอนไซม์เบต้า-กลูโคซิเดสจากพะยูงมีความจำเพาะต่อดัลโคชินินกลูโคไซด์ ขณะที่เอนไซม์ลินามาเรสซึ่งเป็นเอนไซม์เบต้า-กลูโคซิเดสจากมันสำปะหลัง มีความจำเพาะต่อลินามาริน เอนไซม์ดัลโคชิเนสสามารถเร่งปฏิกิริยาย้อนการสลายได้ดี แต่เร่งปฏิกิริยาย้ายหมู่กลูโคสได้ปานกลาง ในทางตรงกันข้ามเอนไซม์ลินามาเรสสามารถเร่งปฏิกิริยาย้ายหมู่กลูโคสได้ดีมาก แต่เร่งปฏิกิริยาย้อนการสลายไม่ได้ งานวิจัยนี้จึงสนใจที่จะศึกษาถึงความสัมพันธ์ระหว่างโครงสร้าง และการทำงานของเอนไซม์เบต้า-กลูโคซิเดส เพื่อหาตำแหน่งของกรดอะมิโนในบริเวณช่องจับกับหมู่อะไกลโคน ที่น่าจะมีความสำคัญต่อความจำเพาะต่อสับสเตรท และการเร่งปฏิกิริยาย้ายหมู่กลูโคส ในงานวิจัยนี้ได้สร้างเอนไซม์ดัลโคชิเนสกลายพันธุ์ 4 ชนิด คือ I185A V255F G367S และ E455I โดยการแทนที่กรดอะมิโนในบริเวณที่คาดว่าทำหน้าที่จับกับหมู่อะไกลโคนของเอนไซม์ดัลโคชิเนส ด้วยกรดอะมิโนของเอนไซม์ลินามาเรส ทำการโคลน และให้มีการแสดงออกในยีสต์ *Pichia pastoris* จากนั้นสกัดให้บริสุทธิ์จากอาหารเลี้ยงเชื้อ จากการศึกษาค่าจลนพลศาสตร์ของเอนไซม์ดัลโคชิเนสกลายพันธุ์ทั้ง 4 ชนิด พบว่าเอนไซม์กลายพันธุ์ I185A V255F G367S และ E455I ไม่ทำให้เอนไซม์สลายลินามารินได้ แต่ลดค่า K_m ในการสลายดัลโคชินินกลูโคไซด์ เอนไซม์กลายพันธุ์ I185A และ E455I ทำให้ค่า K_m ในการสลาย *para*-nitrophenyl-β-D-glucopyranoside (*p*NP-Glc) เพิ่มขึ้น นอกจากนี้เอนไซม์กลายพันธุ์ทั้ง 4 ชนิดทำให้เอนไซม์มีประสิทธิภาพในการสลายดัลโคชินินกลูโคไซด์ และ *p*NP-Glc น้อยลงมาก เมื่อศึกษาการเร่งปฏิกิริยาย้ายหมู่กลูโคสพบว่าเอนไซม์กลายพันธุ์ I185A และ V255F เพิ่มความสามารถในการเร่งปฏิกิริยาย้ายหมู่กลูโคสโดยใช้แอลกอฮอล์ปฐมภูมิ และแอลกอฮอล์ทุติยภูมิเป็นตัวรับ อย่างไรก็ตามเอนไซม์ดัลโคชิเนสกลายพันธุ์ทั้ง 4 ชนิด ยังไม่สามารถเร่งปฏิกิริยาย้ายหมู่กลูโคสโดยใช้แอลกอฮอล์ตติยภูมิเป็นตัวรับได้เลย

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