รหัสโครงการ SUT-102-55-12-34

รายงานการวิจัย

การวิเคราะห์หน้าที่ของเอนไซม์โมโนลิกนอล เบต้า-กลูโคซิเดส จากข้าว

Analysis of Rice Monolignol Beta-Glucosidase Function

ได้รับทุนอุดหนุนการวิจัยจาก

มหาวิทยาลัยเทคโนโลยีสุรนารี

ผลงานวิจัยเป็นความรับผิดชอบของหัวหน้าโครงการวิจัยแต่เพียงผู้เดียว

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คณะผู้วิจัย

หัวหน้าโครงการ

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สิงหาคม 2558

กิตติกรรมประกาศ

การวิจัยครั้งนี้ได้รับทุนอุดหนุนการวิจัยจาก มหาวิทยาลัยเทคโนโลยีสุรนารี ประจำปีงบประมาณ พ.ศ. 2555 ผู้วิจัยขอขอบคุณมา ณ โอกาสนี้

ศาสตราจารย์ ดร. เจมส์ เกตุทัต-คาร์นส์

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บทคัดย่อ

ในพืชชั้นสูงเอนไซม์เบตา-กลูโคซิเดสจัดอยู่ในตระกูลไกลโคไซไฮโดรเลส กลุ่มที่ 1 มีหน้าที่สำคัญหลายอย่าง รวมไปถึงกระบวนการสร้างลิกนิน จากการวิเคราะห์ phylogenetic ของเอนไซม์เบตา-กลูโคซิเดสในข้าว พบว่า Os4BGlu14 Os4BGlu16 และ Os4BGlu18 มีลำดับกรดอะมิโนที่มีความใกล้เคียงกับเอนไซม์ในกลุ่มโมโนลิกนอลเบตา-กลูโคซิเดสมากที่สุด จึงนำมาสู่สมมติฐานว่าเอนไซม์ทั้ง 3 ตัวนี้เกี่ยวข้องกับกระบวนการสร้างลิกนิน อย่างไรก็ตาม Os4BGlu14 ไม่มีกรดอะมิโนกลูตาเมทในตำแหน่ง catalytic acid/base ดังนั้น Os4BGlu14 อาจเป็นเอนไซม์ที่ไม่สามารถทำงานได้เหมือนไกลโคไซไฮโดรเลส กลุ่มที่ 1 ทั่วไป ยีน Os4BGlu16 ถูกสังเคราะห์ขึ้นเพื่อใช้ในการผลิตเอนไซม์ในยีสต์ เอนไซม์ผสม Os4BGlu16 ที่ถูกผลิตและแยกให้บริสุทธิ์จากอาหารที่ใช้เลี้ยง Os4BGlu16 ด้วยวิธี immobilized metal affinity chromatography (IMAC) ในทางกลับกัน Os4BGlu18 สามารถผลิตใน *Escherichia coli* โดยมี thioredoxin และ His_6 tags ต่อที่ปลาย N-terminal และนำมาแยกให้บริสุทธิ์ด้วย anion exchange chromatography hydrophobic interaction chromatography และ IMAC column ตามลำดับ เอนไซม์ Os4BGlu16 และ Os4BGlu18 สามารถย่อยโมโนลิกนอลกลูโคไซด์สับสเตรท coniferin syringin และ p-coumarol glucoside ได้อย่างมีประสิทธิภาพมากกว่าสับสเตรทตัวอื่น

ในการตรวจหาการแสดงออกของยีนโมโนลิกนอลเบตา-กลูโคซิเดสในข้าว พบว่า mRNA ของ Os4BGlu14 มีการตรวจพบมากที่สุดในส่วนของเมล็ด รวง และเกสร ส่วน mRNA ของ Os4BGlu16 พบมากที่สุดในส่วนของใบจากต้นข้าวสัปดาห์ที่ 4 ถึงสัปดาห์ที่ 10 เอนโดสเปิร์ม และเปลือกเมล็ดส่วนนอก และ mRNA ของ Os4BGlu18 ส่วนใหญ่ถูกพบในช่วงแรกของการเจริญเติบโตตั้งแต่สัปดาห์แรกถึงสัปดาห์ที่ 4 และยังถูกพบในเกสรและเปลือกเมล็ดส่วนนอกอีกด้วย ข้อมูลเหล่านี้บ่งบอกถึงการทำงานของเอนไซม์โมโนลิกนอลเบตา-กลูโคซิเดสเกิดขึ้นทั้งในช่วงเจริญเติบโตจนถึงช่วงออกผลผลิต

Abstract

Plant glycoside hydrolase family 1 (GH1) β-glucosidases have been implicated in several functions, including lignification. In phylogenetic analysis of rice (*Oryza sativa* L.) GH1 β-glucosidases, Os4BGlu14, Os4BGlu16, and Os4BGlu18 grouped closely with known monolignol β-glucosidases, leading to the hypothesis that they may release monolignols from their inactive glucosides. However, Os4BGlu14 lacks the conserved glutamate in the acid/base position found in other GH1 β-glucosidases, so it may not be active. An optimized Os4BGlu16 cDNA was synthesized for expression of the protein in *Pichia pastoris*. Os4BGlu16 fusion protein was purified from induced *P. pastoris* culture media by immobilized metal affinity chromatography (IMAC) to yield a single prominent protein band on SDS-PAGE analysis and strong β-glucosidase activity. In contrast, active Os4BGlu18 β-glucosidase fusion protein with N-terminal thioredoxin and His_6 tags was successfully expressed and extracted from *E. coli* cells, and was purified by anion exchange chromatography, hydrophobic interaction chromatography and IMAC. Os4BGlu16 and Os4BGlu18 hydrolyzed the monolignol glucosides coniferin, syringin, and *p*-coumarol glucoside with much higher catalytic efficiencies than other substrates.

By quantitative RT-PCR, highest Os4BGlu14 mRNA levels were detected in seed, panicle and pollen. Os4BGlu16 was detected at highest levels in leaf from 4 to 10 weeks, endosperm and lemma, while Os4BGlu18 mRNA was most abundant in vegetative tissues from 1 week to 4 weeks old and in pollen and lemma. These data suggest a role for monolignol β-glucosidases in both vegetative and reproductive rice tissues.

สารบัญ

หน้า

สารบัญ (ต่อ)

หน้า

สารบัญรูปภาพ

สารบัญตาราง

บทที่ 1
บทนำ

1.1 ความสำคัญและที่มาของปัญหาที่ทำการวิจัย

Rice is a major part of Thai society and economy and has impact on the ecology as well. Rice is the most important crop in Thailand, leading in both local consumption and export, as well as in social and psychological meaning. Despite the fact that Thailand is one of the leading exporters of rice, rice production in Thailand is inefficient with a low yield per hectare. Although this may be largely attributed to abiotic stresses, like drought and soil salinity, the effects of biotic stress, especially rice blast, are also serious constraints. Therefore, research to learn more about rice and its response to fungal invasion and application of that knowledge to the production of rice has great potential for the economic and social advancement of the Thai people.

Though the selective approach of breeding rice, particularly with the advance of molecular marker assisted breeding, has made great strides in improving rice for various challenges facing Thailand, this somewhat random approach to identifying genes to select can be improved by the application of knowledge about rice biochemistry. Since rice, like all plants, has a wide variety of carbohydrates, including polysaccharides, glycosides, glycolipids and glycoproteins that make up a large portion of the mass and play critical functional roles. As fungal blast, and other microbial diseases like bacterial blight contribute to the low yields of rice in Thailand, knowledge of the roles of beta-glycosidases that act in the production of lignin and antimicrobial lignans in response to microbial invisions is expected to help produce higher yielding rice in the future.

Rice has an unfortunate environmental outcome due to the current practice of burning much of the rice straw and husk to remove it from the fields, since the amount produced is too much to be naturally degraded over the course of the year. This creates a pollution problem, including introducing carcinogenic forms of silica into the air, in addition to soot and carbon dioxide that contributes to global warming. Currently, there is a great interest in generating biofuels, including ethanol, from plant

products. Unfortunately, much of the emphasis in Thailand has been on starch production, the crops for which are grown at the expense of food crop land or destroying the forests, which may generate a net gain in carbon dioxide production and degrading the soil. The production of ethanol from cellulosic wastes, like rice husk and straw, would be of much greater benefit, since no additional land need be converted to agriculture and less pollution is generated than if they are burned. Alternatively, there is much interest in developing high yield grasses that can grow on marginal soil as a source of biomass for fuel production. Currently, production of ethanol from cellulosic wastes is generally not economical due to the input of energy and other resources. Improved processes are expected to lead to a net gain in the next few years, but the process can benefit from improved enzymes, as well as other processes. In some biofuel production processes, such as release of glucose from lignocellulose, low lignin content is desirable, while in other fuel production processes a high content is desirable. Therefore, it would be useful to be able to modulate lignin content in the cell walls of grasses, especially if this can be done without greater microbial susceptibility of the plants. However, this must be done without sacrificing the defensive fitness of the plant to resist various pests, which requires a strong knowledge of enzymes that modulate lignin and lignin content. So, this project also has potential to contribute to biofuel production and to help improve the environment for Thailand and the world. Also, since similar compounds have antimicrobial functions, the products may have application in this area as well.

In addition to the potential economic and environmental benefits from the knowledge gained from this project, there are several benefits from the academic output. First, a student complete a part of her Ph.D. thesis on this project, so Thailand will gain in the quality of human resources for biotechnology.

1.2 วัตถุประสงค์ของการวิจัย

1) To clone and express the putative rice monolignol β-glucosidases, optimize for suitable expression conditions, and purify proteins.
2) To characterize the enzymatic properties and substrate specificities of recombinant Os4BGlu16 and Os4BGlu18 enzymes, including activities

toward monolignol substrates, *p*NP-glycosides, natural glycosides and oligosaccharides.

3) To investigate the relative expression of monolignol β-glucosidase gene in different rice tissues.

1.3 ขอบเขตของการวิจัย (Scope of research)

In this project, characterized the activity of Os4BGlu16 that is expressed in the yeast *Pichia pastoris* and or the bacteria *Escherichia coli* toward monolignol glycosides and other natural and synthetic glycosides. Although Os4BGlu18 had previously been expressed and partially characterized, the protein expression was optimized and the purified protein tested against monolignoal glucosides and other natural substrates. These were tested by both TLC and LC-MS of the extracts with and without enzyme treatment. Initially, we had planned to test for natural substrates in plant extracts as well, but since the monolignol glucosides were hydrolyzed with high efficiency, there seemed no need to identify additional substrates.

In collaboration with Prof. Tom Okita and Prof. Norman Lewis of the Institute of Biological Chemistry of Washington State University, we planned to complete the production of plants with knockdowns of Os4BGlu14, Os4BGlu16 and Os4BGlu18 to identify any phenotypes, in terms of plant appearance, lignin and lignan distribution and metabolite profiles. However, technical difficulties prevented completion of this part of the project.

1.4 ทฤษฎี สมมุติฐาน และกรอบแนวความคิดของการวิจัย (Theory and strategy of research)

Aside from being the world's most important food crop, rice is also a model plant for monocot species with a sequenced genome (International Rice Genome Project, 2005). The availability of the complete genome allows exploration of the biochemical and biological functions of genes of potential interest. Aside from the wide range of polysaccharides, oligosaccharides, and complex carbohydrates, plants, including rice, contain an array of glycosides with aryl and alkyl aglycones that play roles in signaling in development and response to stress. Carbohydrate active enzymes, as catalogued in the CAZY website (www.cazy.org; Cantaral et al., 2009) regulate the activity of these

compounds, particularly glycoside hydrolases and glycosyltransferases. Lignin is produced from monolignols, which are often stored as glucosides in the vacuoles of plant cells. Although glycosylation of monolignols is apparently not critical to lignification of Arabidopsis cell walls, a beta-glucosidase that deglycosylates the monolignol glucoside conifierin have been identified in cell walls near the site of lignifications in pine tree. However, the function of monolignol β-glucosidases and monolignol glycosides was not previously explored in monocots, such as grasses, which are promoted as one of the major feed stocks for cellulosic biofuel production.

In rice, there are three GH1 genes that produce proteins that are closely related to the Arabidopsis beta-glucosidases that hydrolyze monolignol glucosides and pine tree coniferin beta-glucosidase, which are Os4BGlu14, Os4BGlu16, and Os4BGlu18. We explored the functions of these enzymes, both in our laboratory and in collaboration with Prof. Tom Okita at Washington State University via an RGJ grant. In our laboratory, we had expressed the Os4BGlu18 beta-glucosidase and done a preliminary characterization of its activity, which showed that it hydrolyzes *p*-coumaryl alcohol glucoside, but we could not detect hydrolysis of other monolignol glucosides. Initial attempts were made to express and characterize Os4BGlu14 and Os4BGlu16, but the sequence of Os4BGlu14 lacks the conserved catalytic acid/base, so it is not likely to be a monolignol β-glucosidase. Therefore, we would planned to express and characterize Os4BGlu16 and do more characterization of Os4BGlu18 with substrates that had not yet been tested. In this project, in collaboration with Prof. Okita, we also developed antibodies against Os4BGlu14, Os4BGlu16 and Os4BGlu18 to identify the localization of these enzymes in the plant and recombinant protein expression systems. This will help to determine whether they are likely to act in monolignol activation at the cell wall or have a different function. Knowledge of the function of these rice β-glucosidases may allow manipulation of their expression to make rice straw a better feed stock for alcohol production, as well as potentially improving rice production.

1.5 ประโยชน์ที่ได้รับจากงานวิจัย

1) Benefits in addressing the problems of the institution:

This research benefited SUT by providing for graduate student training and material for international publications and meeting presentations to improve the standing of SUT in the academic community. This work contributed to an international publication and also to a student's thesis. It was presented at an international symposium as well.

2) Generation of new knowledge

This work allowed us to identify the substrates that these enzymes hydrolyze and their products, and to study the level of gene expression in different stages in order to better understand the enzyme functions.

3) Providing knowledge to the people.

This project was provided research training to a graduate student and a research assistant. It was provided important knowledge of the metabolism of rice plants, which may be used to improve the utilization of rice straw or improved rice as a food crop in the future. The functions of these enzymes are determined and this knowledge is of obvious application to the growth of rice or rice straw utilization, after scientific publication, the knowledge was spread to the general media, as well as scientific media.

4) Production of knowledge for business.

In this project, we have two aspects of potential use to businesses in the future. The new knowledge of rice metabolism will help for knowledge-based breeding efforts in the future to generate higher value crops.

5) Use in improving product quality

The knowledge used for production of higher quality rice seed in the future. Currently, it provided for high quality graduates of SUT.

6) Benefits to target groups.

Scientists were gained new knowledge in the role of monocot monolignol β-glucosidases in the modulation of lignin and other plant metabolites.

บทที่ 2

วิธีดำเนินการวิจัย

2.1 Amplification and cloning of monolignol β-glucosidases

2.1.1 Cloning of optimized Os4BGlu16 cDNA into the pPICZαB(NH8) vector

An optimized Os4BGlu16 cDNA (protein accession number Q7XSK2) was synthesized and inserted into the pUC57 vector by GenScript Corporation (Piscataway, NJ, USA). The optimized Os4BGlu16 cDNA was transformed into XL1-Blue competent cells and spread onto LB agar containing 50 μg/ml ampicillin. The colonies were picked and inoculated into LB media containing 50 μg/ml ampicillin and incubated at 37 °C overnight. The plasmid was extracted with a QIAprep Spin miniprep Kit (QAIGEN, CA, USA). The optimized Os4BGlu16 and pPICZαBNH8 plasmid (Toonkool et al., 2006) were cut with *Pst*I and *Xba*I. The gel purified Os4BGlu16 insert and pPICZαBNH8 plasmid were ligated as described for the cloning of Os4BGlu14 and colonies selected on 25 μg/ml zeocin. The recombinant gene insert was checked by cutting the plasmid clones with *Pst*I and *Xba*I and evaluating the products by agarose gel electrophoresis, and the sequence confirmed by automated DNA sequencing at Macrogen Corp. (Seoul, Korea).

2.1.2 Amplification and cloning of cDNA encoding mature Os4BGlu18

The gene encoding full-length rice Os4BGlu18 was amplified from the cDNA synthesis product with the Os4BGlu18Startf and Os4BGlu18Stop primers. Then, the gene encoding the predicted mature protein was also amplified from the initial PCR product with the Os4BGlu18MatStf and Os4BGlu18Stop primers. Both reactions were carried out with *Pfu* DNA polymerase and PCR product (~1.5 kb) was checked by electrophoresis on a 1% agarose gel.

The PCR mixture that contained the mature Os4BGlu18 gene and pET32a expression vector were digested with *Nco*I and *Bam*HI at 37 °C for 2 h. The digested Os4BGlu18 gene and pET32a vector were purified with the HiYield™ Gel/PCR DNA fragments extraction kit. The purified Os4BGlu18 gene and pET32a vector were ligated together by mixing 1:3 vector:DNA insert and heating at 45 °C for 5 min

to melt any cohesive termini that have reannealed, then immediately chilled on ice. Then, 1X ligation buffer and T4 DNA ligase were added and the tube was mixed well but gently and incubated at 15 °C for 18 h. One microliter of the reaction was transformed into DH5α competent cells and selected on a 50 μg/ml ampicillin LB agar plate. The colonies were cultured and plasmids prepared as described for the pET32/Os4BGlu14 plasmid. The recombinant gene insert was checked by cutting with *Nco*I and *Bam*HI and the sequence verified by automated DNA sequencing at Macrogen Corp. (Seoul, South Korea).

2.2 Expression of monolignol β-glucosidases

2.2.1 Expression and purification of Os4BGlu16

The optimized pPICZαBNH8/Os4BGlu16 plasmid was linearized with *Sac*I and transformed into *P. pastoris* competent cells by electroporation (*Pichia* manual, Invitrogen). The transformed cells were selected on Yeast Extract Peptone Dextrose medium with Sorbitol (YPDS) plates containing 100 μg/ml zeocin at 28 °C for 3-5 days. The transformed cells were selected again on a YPDS plate containing 500 μg/ml zeocin. The colonies were screened for protein production in small scale cultures. For protein production, a single colony that has been selected on a 500 μg/ml zeocin YPDS plate was inoculated into 500 ml of buffered glycerol-complex medium (BMGY) medium containing 100 μg/ml zeocin and grown in a shaking incubator (220 rpm) at 28 °C until the cell culture optical density at 600 nm (OD_{600}) reached 2-3. The cells were harvested by centrifugation and resuspended in 1000 ml buffered methanol-complex medium (BMMY) at the final OD_{600} of 1. Protein expression was induced by adding methanol to 1% (v/v) final concentration every 24 h for 4 days at 20 °C with shaking, and the media was checked each day for activity (Luang et al., 2010).

The protein was purified from the culture broth after removal of the cells by centrifugation. The pH of the culture broth with secreted protein was adjusted to 7.5 with 2 M K_2HPO_4 and it was loaded onto immobilized metal ion affinity chromatography (IMAC) column (GE Healthcare, Buckinghamshire, United Kingdom) charged with Co^{2+}, and the column was washed with 5 column volumes of 5 mM and 10 mM of imidazole in 50 mM sodium phosphate buffer, pH 7.5, then the

protein was eluted with 250 mM imidazole in 50 mM sodium phosphate buffer, pH 7.5. The active fractions were reconstituted by centrifugal filtration.

To test for the significance of glycosylation, the purified protein was deglycosylated with endoglycosidase H (New England BioLabs, MA, USA). The mixture of 90 µg Os4BGlu16 enzyme and 500 U endoglycosidase H in 50 mM sodium acetate, pH 5.5, was incubated at 4 °C for 3-4 days with gentle shaking, until deglycosylation was completed, based on inspection on SDS-PAGE.

2.2.2 Expression and purification of Os4BGlu18

The recombinant pET32a/Os4BGlu18 plasmids was transformed into Origami(DE3) competent cells and spread onto LB-agar containing 50 µg/ml ampicillin, 15 µg/ml kanamycin and 12.5 µg/ml tetracycline. The plate was incubated at 37 °C overnight. The colonies that grew overnight were picked and inoculated into LB media containing the same antibiotics to make a starter culture. To express the recombinant Os4BGlu18 β-glucosidases, 1% final concentration of starter culture was added into the same type of media and cultured at 37 °C with rotary shaking at 200 rpm. Protein expression was induced when the OD_{600} of the culture reached 0.6. The protein expression was induced with 0.1 mM IPTG at 18 °C for 16-18 h. The cell pellets were collected by centrifugation at 4,500 rpm 15 min at 4 °C. The cell pellets was kept at -80 °C to allow freeze-thaw breakage before use.

The IPTG-induced bacterial cell pellets were thawed on ice and then resuspended in freshly prepared extraction buffer (20 mM Tris-HCl buffer, pH 8.0, 200 µg/ml lysozyme, 1% Triton-X 100, 1 mM PMSF, 25 µg/ml DNase I and 0.1 mg/ml soy bean trypsin inhibitor) in a ratio of 5 ml extraction buffer per gram fresh weight of cell pellets. The resuspended cells were incubated at room temperature for 30 min. Then, the insoluble proteins and cell debris were removed by centrifugation.

The supernatant fraction was loaded onto a Q sepharose chromatography (GE Healthcare) column, which had been pre-equilibrated with 50 mM Tris-HCl buffer, pH 8.0, and run at a flow rate of 2 ml/min. Unbound protein was washed out with 2 CV of 50 mM Tris-HCl buffer, pH 8.0. The protein was eluted by a gradient from 0-1 M NaCl in 50 mM Tris-HCl buffer, pH 8.0. The active protein was pooled and NaCl was added to 2 M final concentration. The protein solution was then loaded onto a phenyl sepharose chromatography (GE Healthcare) column, which was pre-

equilibrated with 2 M NaCl in 50 mM Tris-HCl, pH 8.0. The column was washed with 2 CV of 2 M NaCl in 50 mM Tris-HCl, pH 8.0, and eluted with gradient from 2-0 M NaCl in 50 mM Tris-HCl, pH 8.0, and further eluted with 0-60% ethylene glycol in 50 mM Tris-HCl, pH 8.0. The fractions that contained β-glucosidase activity were pooled and concentrated, and the buffer was changed to 50 mM Tris-HCl buffer, pH 8.0, in 30 kDa molecular weight cut-off (MWCO) Amicon® Ultra-15 centrifugal filter. Finally, active Os4BGlu18 protein from the previous column was loaded into an immobilized metal affinity chromatography (IMAC) column charged with Co^{2+}. After loading the active protein, the IMAC column was washed twice with 10 CV of equilibration/wash buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl) to remove unbound protein and washed again with 5 CV each of equilibration/wash buffer containing 5 mM and 10 mM of imidazole, respectively. Bound protein was eluted with 3 CV of elution buffer (50 mM Tris-HCl, pH 8.0, containing 250 mM imidazole). The eluted protein was checked by assaying activity with *p*NPGlc and the presence and purity of protein of appropriate size evaluated by SDS-PAGE. The fractions that contained β-glucosidase were pooled and imidazole removed by buffer exchange in 30 kDa MWCO Amicon® Ultra-15 centrifugal filters.

2.3 Enzyme assay, pH and temperature optimum and stability studies

Initially, 1 µg of purified protein or 5–50 µl of purification fraction was incubated in 140 µl total volume with 1 mM pNPGlc in 50 mM sodium acetate, pH 5.0, at 30 °C for 30 min, and then 70 µl of 2 M Na_2CO_3 was added to stop the reaction. The absorbance was measured at 405 nm and compared to a *p*-nitrophenolate (pNP) standard curve in the same buffers to determine the amount of *p*NP released. Upon determination of the pH optima, Os4BGlu16 was assayed in 50 mM sodium phosphate, pH 6.5, and Os4BGlu18 in 50 mM sodium acetate, pH 5.0.

To determine the pH optima of the purified protein, activity was assayed in 100 mM universal buffers (citric acid–disodium hydrogen phosphate) ranging from pH 2.0 to 9.0, at 0.5 pH unit increments. The pH stability for the purified enzyme was determined by incubating the enzymes in 100 mM buffers ranging from pH 2.0 to 9.0 for 15, 30, 60 min and 2 h at room temperature (~25 °C). After incubation, the

enzyme was assayed in 50 mM buffer at the optimum pH and 1 mM pNPGlc, as described above.

The optimum temperature for enzyme activity was determined by pre-incubating the purified enzyme at temperatures ranging from 5 °C to 70 °C at 5 °C increments for 10 min, and then pre-incubated enzyme was incubated with 1 mM *p*NPGlc in 50 mM buffer at the optimum pH, in a reaction volume of 140 µl at the same temperatures for 30 min, and then 70 µl of 2 M Na_2CO_3 was added to stop the reaction, the enzyme activity was measured, as described above. Thermostability of the purified enzyme was measured by incubating the enzyme in 50 mM buffer at different temperatures in the range of 20 °C to 60 °C at 10 °C increments for 15, 30, 45, 60 min, then the enzyme samples was assayed with 1 mM *p*NPGlc in 50 mM sodium acetate, pH 5.0, at 30 °C for 30 min.

2.4 Substrate specificity and enzyme kinetics

The substrate specificity toward natural and synthetic substrates was evaluated by incubating 1 µg of enzyme with 1 mM final concentration of substrates at 30 °C for 1 h in 100 mM sodium phosphate, pH 6.5, for Os4BGlu16 or 100 mM sodium acetate, pH 5.0, for Os4BGlu18. Hydrolysis of *p*NP- and *ortho*-nitrophenyl (*o*NP) glylcosides was detected as described for *p*NPGlc above. Reactions with other synthetic and natural glucosides were stopped by boiling for 5 min and the amount of glucose released was determined by peroxidase/glucose oxidase coupled reactions (PGO assay, Sigma Aldrich) or the product from the hydrolysis reaction was spotted onto thin-layer chromatography silica gel 60 F254plates (Merck, Darm-stadt, Germany). TLC plates were developed with a solvent of ethyl acetate, methanol and water (7:2.5:1, v/v/v) and detected by spraying with 10% sulfuric acid in methanol, drying and heating at 120 °C.

Apparent kinetic parameters, K_M and V_{max}, of purified protein with *p*NP-glycosides and natural and synthetic substrates were determined in triplicate reactions. The optimum time point, at which the velocity of hydrolysis gives a first order rate constant, was determined. The rates were determined over a substrate concentration range of at least 0.2 K_M to 3 K_M. Kinetic parameters were calculated by fitting the rate

of product formation and substrate concentrations by nonlinear regression of the Michaelis-Menten curves with Grafit 5.0 (Erithacus Software, Horley, Surrey, U.K.). The apparent k_{cat} values were calculated by dividing the V_{max} by the total amount of protein in the reaction.

For monolignol substrates, glucose released was measured by high performance anion exchange chromatography on a Dionex ion chromatography system (ICS-3000). The boiled reactions were diluted 100-fold with HPLC grade water and loaded onto a Dionex CarboPac™ PA20 carbohydrate column (3 × 150 mm), which was then eluted with 10 mM NaOH in water. The peak areas were compared to a glucose standard curve to determine the amount of glucose released. Since the substrate concentrations in these assays did not bracket the K_M for accurate nonlinear regression, the kinetic parameters were calculated by linear regression of the Hanes-Woolf ([S]/v vs. [S]) plot, which produced a straight line with slope $1/V_{max}$ and a y-intercept of K_M/V_{max} and an x-intercept of $-K_M$ (Haldane, 1957).

2.5 *In planta* expression analysis of rice monolignol β-glucosidase

Total RNA was isolated from tissues of various age rice plants with the Spectrum™ plant total RNA kit (Sigma-Aldrich, MO, USA). Subsequently, 100 ng/μl of total RNA was mixed with 2.5 μM oligo$(dT)_{20}$ and 200 U Superscript™III reverse transcriptase (Invitrogen, CA, USA), and the mixture was reverse transcribed at 50 °C for 50 min. Real-time PCR was done with the gene-specific primers listed in Table 2.1. The PCR reaction included SYBR Green Mix (KapaBiosystems, Boston, MA, USA), 1st strand cDNA, the gene specific primer pair and *Taq* polymerase, along with deoxyribonucleotides and buffer. After the PCR finished, the products were examined by 2% agarose gel to verify specificity. Relative gene expression ratios (rERs) were calculated from the C_T values and efficiency, E, with a 7-day-old shoot cDNA as the reference sample and actin as the reference gene (Schefe et al., 2006).

บทที่ 3

Results (ผลการวิจัย)

3.1 Selection of Os4BGlu16 and Os4BGlu18 for expression and characterization

The Os4BGlu14, Os4BGlu16, and Os4BGlu18 predicted proteins from the rice genome are similar to monolignol beta-glucosidases from pine tree and Arabidopsis (Figure 3.1), but they had not previously been characterized to hydrolyze monoligonol glucosides. The cDNA encoding the mature Os4BGlu14 protein (AK067841, Kikuchi et al., 2003) was cloned into the pET32a expression vector, and used to express the protein in *E. coli*, but no activity was detected under any conditions tested. Because the catalytic acid/base of Os4BGlu14 is replaced by glutamine residue 191 (Figure 3.1), it was expected that it might not have activity, but so a cDNA with the mutation of pET32a/Os4BGlu14Q191E was made by site-directed mutagenesis. However, neither the wild type nor the mutant construct yielded enzyme hydrolyzing pNPGlc, oNPGlc, or 2,4-dNPGlc substrates in any conditions tested (pET32a expression of thioredoxin-Os4BGlu14 protein in *E. coli* strains Origami(DE3), Origami B(DE3) or Rosetta-gami(DE3) at various temperatures and IPTG inducer concentrations). Therefore, we concentrated on expression and characterization of Os4BGlu16 and Os4BGlu18.

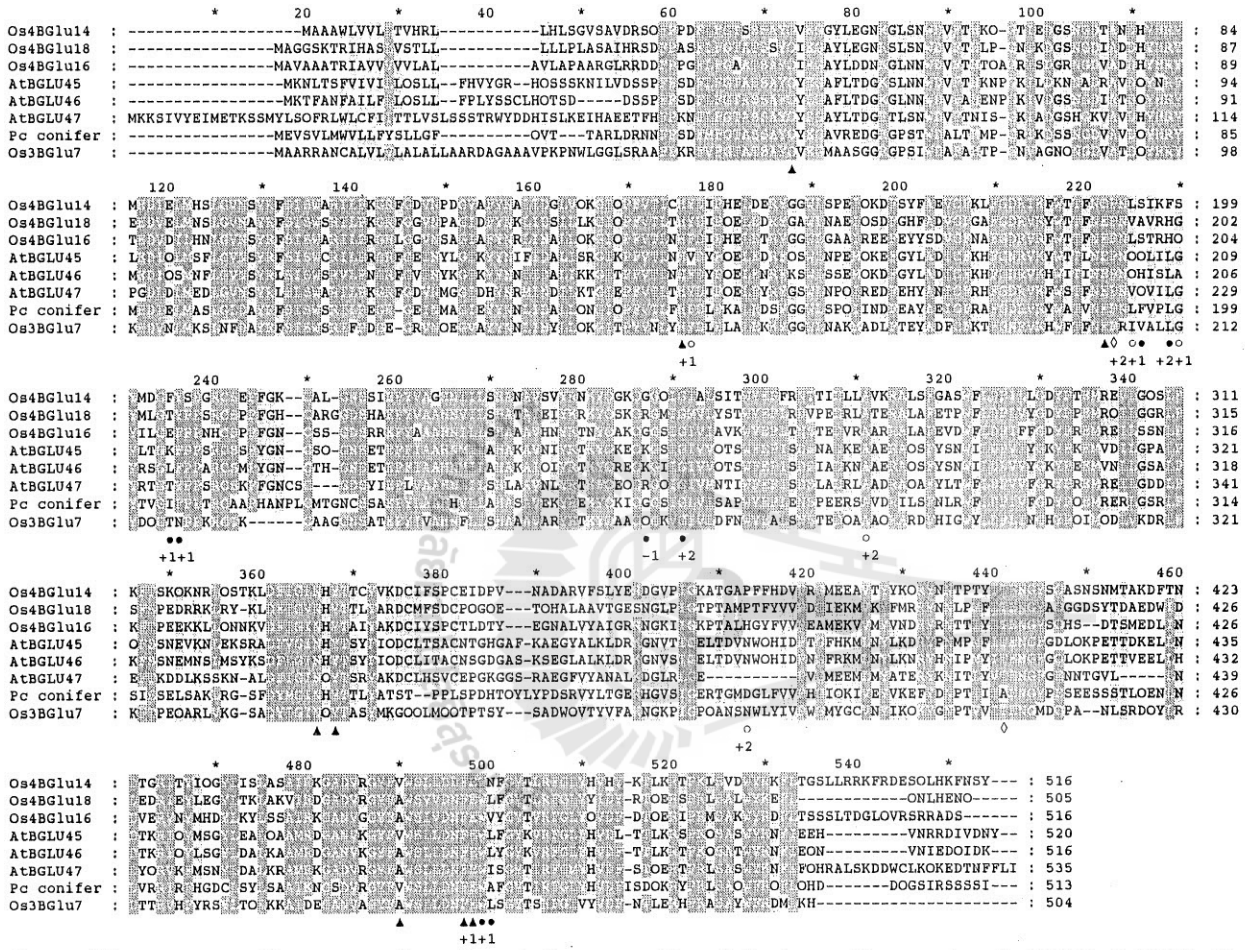


Figure 3.1 Amino acid sequence alignment of predicted rice monolignol β-glucosidases, rice Os3BGlu7 (BGlu1), *Arabidopsis thaliana* β-glucosidases (BGLU45, BGLU46, BGLU47) and *Pinus contorta* coniferin β-glucosidase.

3.2.Cloning and expression of Os4BGlu16

A gene optimized for Os4BGlu16 (Genbank accession number KJ579205) expression in *Pichia pastoris* was synthesized and inserted into the pUC57 vector by GenScript Corporation (Piscataway, NJ, USA). The optimized Os4BGlu16 cDNA was inserted into the pPICZαBNH$_8$ plasmid (Toonkool et al., 2006).

The pPICZαBNH$_8$/Os4BGlu16 plasmid was cloned into *P. pastoris* strain SMD1168H, and protein expression was induced with 1% (v/v) methanol for 7 days at 20 °C. Of the twenty clones examined for *p*NPGlc hydrolysis activity in the media, Clone 6 had the highest activity, which was maximal at 4 days induction (Figure 3.2).

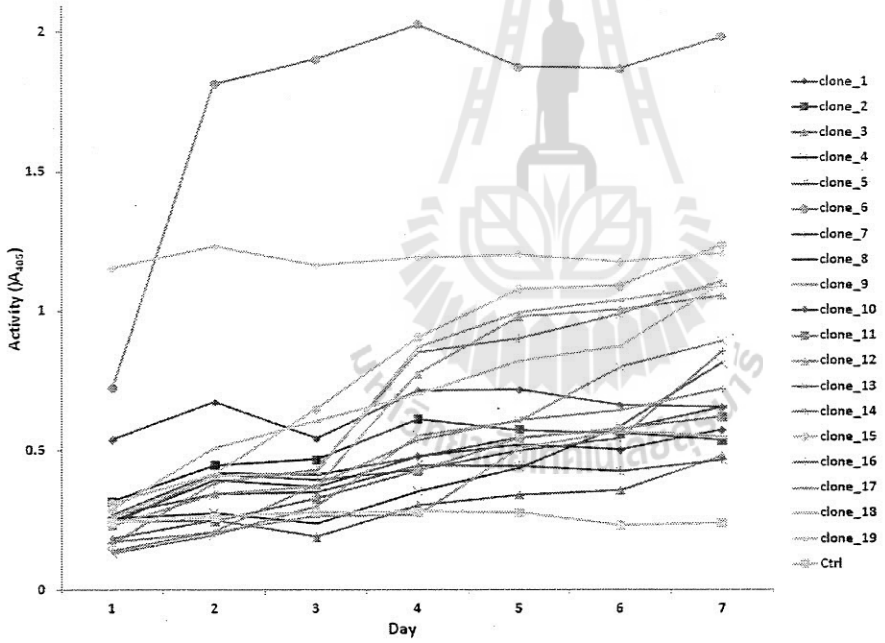


Figure 3.2 The *p*NPGlc hydrolysis activity in *pichia* media over 7 days of expression of clones expressing Os4Glu16. The activity was determined by incubating 50 µl of induced cells media with 1 mM *p*NPGlc, in 50 mM NaOAc, pH 5.0, at 30 °C for 30 min. The OD$_{405}$ is plotted versus the day.

3.3. Optimization of expression of Os4BGlu18

The cDNA encoding mature rice Os4BGlu18 was amplified by reverse transcription and nested PCR with total mRNA of 7-day-old rice (cv.KDML105) roots and shoots as template. A single DNA band of mature Os4BGlu18 gene was amplified by PCR and cloned into the pET32a(+) expression vector.

Expression of Os4BGlu18 was attempted with the recombinant pET32a/Os4BGlu18 plasmids in *E. coli* strains Origami(DE3), Origami B(DE3), and Rosetta-gami(DE3) by inducing with 0.4 mM IPTG at 20 °C for 16 h (Figure 3.3). The activity of this enzyme could detected in soluble cell lysates of Origami(DE3) and Origami B(DE3) with pNPGlc substrate at similar levels, thus, Origami(DE3) was chosen as host cells for Os4BGlu18 expressions. The optimization of expression temperature was varied from 10 °C to 22 °C (Figure 3.11) with 18 °C found to give highest activity with pNPGlc substrate. Then, the concentration of IPTG used for induction was varied from 0 to 0.8 mM at 18 °C for 16 h (Figure 3.4). Os4BGlu18 enzyme could be expressed at all concentrations of IPTG. However, cells induced with 0.1 mM IPTG had the highest activity when Os4BGlu18 enzyme activity was tested with *p*NPGlc. In the absence of IPTG, the activity detected was only 44% compared with 0.1 mM IPTG induced cells. At 0.2-0.8 mM IPTG induction, the activity decreased 10-30%. Os4BGlu18 required at least 16 h to 24 h for induction. After induction for 12 h, the activity detected was only 28% of that at 16 h. Finally, the *E.coli* strains Origami(DE3) was used to express Os4BGlu18 enzyme and induced with 0.1 mM IPTG at 18 °C.

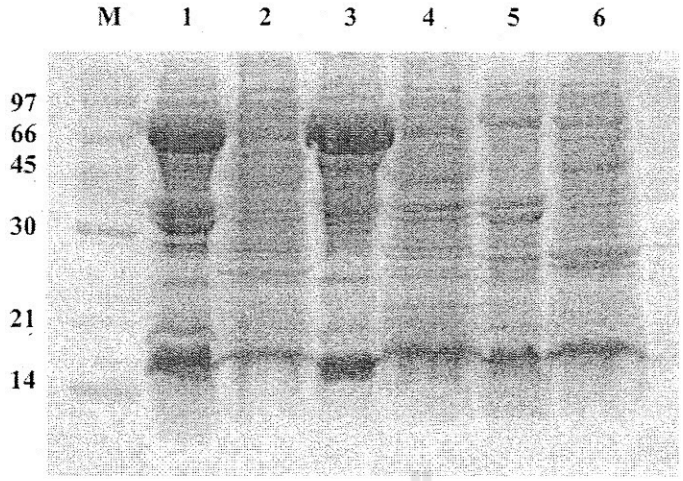


Figure 3.3 SDS-PAGE analysis of pET32a/Os4BGlu18 expression in various *E. coli* host cells. The cells were induced with 0.4 mM IPTG at 20 °C for 16 h.

Lane M, Bio-Rad low molecular weight markers; Lane 1, insoluble protein from Origami(DE3); Lane 2, soluble protein from Origami(DE3); Lane 3, insoluble protein from Origami B(DE3); Lane 4, soluble protein from Origami B(DE3); Lane 5, insoluble protein from Rosetta-gami(DE3); Lane 6, soluble protein from Rosetta-gami(DE3).

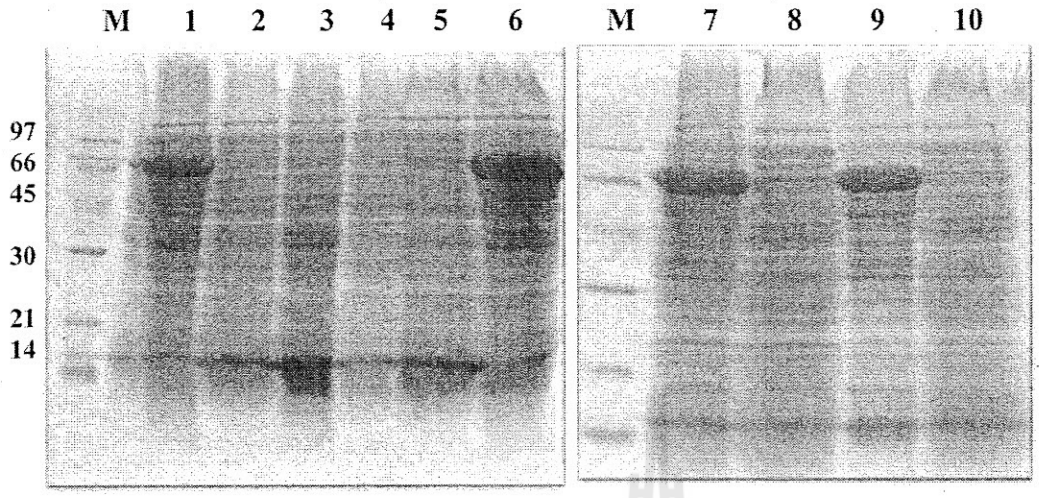


Figure 3.4 SDS-PAGE analysis of pET32a/Os4BGlu18 expression in Origami(DE3) at temperatures varying from 10-22 °C and induced at 0.4 mM IPTG for 16 h.

Lane M, Bio-Rad low molecular weight markers; Lane 1, insoluble protein from pET32a/Os4BGlu18 culture at 10 °C; Lane 2, pET32a/Os4BGlu18 soluble protein at 10 °C; Lane 3, pET32a/Os4BGlu18 insoluble protein at 15 °C; Lane 4, pET32a/Os4BGlu18 soluble protein at 15 °C; Lane 5, pET32a/Os4BGlu18 insoluble protein at 18 °C; Lane 6, pET32a/Os4BGlu18 soluble protein at 18 °C; Lane 7, pET32a/Os4BGlu18 insoluble protein at 20 °C; Lane 8, pET32a/Os4BGlu18 soluble protein at 20 °C; Lane 9, pET32a/Os4BGlu18 insoluble protein at 22 °C; Lane 10, pET32a/Os4BGlu18 soluble protein at 22 °C.

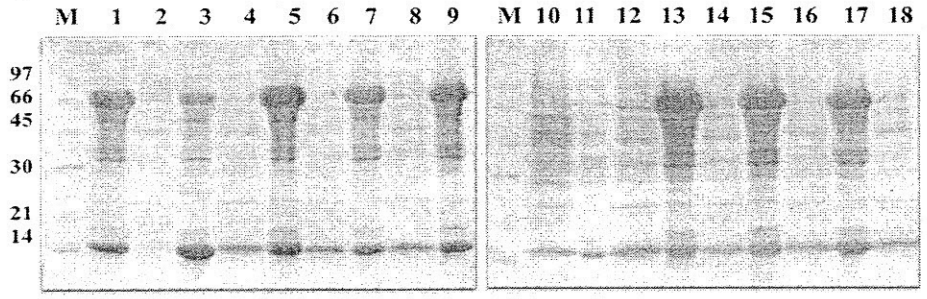


Figure 3.5 SDS-PAGE analysis of pET32a/Os4BGlu18 expression in Origami(DE3) induced at 0 to 0.8 mM of IPTG. Cultures were induced at 18 °C for 16 h.

Lane M, Bio-Rad low molecular weight markers; Lane 1, 0 mM IPTG pET32a/Os4BGlu18 culture soluble protein; Lane 2, 0 mM IPTG pET32a/Os4BGlu18 culture insoluble protein; Lane 3, 0.1 mM IPTG pET32a/Os4BGlu18 culture soluble protein; Lane 4, 0.1 mM IPTG pET32a/Os4BGlu18 culture insoluble protein; Lane 5, 0.2 mM IPTG pET32a/Os4BGlu18 culture soluble protein; Lane 6, 0.2 mM IPTG pET32a/Os4BGlu18 culture insoluble protein; Lane 7, 0.3 mM IPTG pET32a/Os4BGlu18 culture soluble protein; Lane 8, 0.3 mM IPTG pET32a/Os4BGlu18 culture insoluble protein; Lane 9, 0.4 mM IPTG pET32a/Os4BGlu18 culture soluble protein; Lane 10, 0.4 mM IPTG pET32a/Os4BGlu18 culture insoluble protein; Lane 11, 0.5 mM IPTG pET32a/Os4BGlu18 culture soluble protein; Lane 12, 0.5 mM pET32a/Os4BGlu18 culture insoluble protein; Lane 13, 0.6 mM IPTG pET32a/Os4BGlu18 culture soluble protein; Lane 14, 0.6 mM IPTG pET32a/Os4BGlu18 culture insoluble protein; Lane 15, 0.7 mM IPTG pET32a/Os4BGlu18 culture soluble protein; Lane 16, 0.7 mM IPTG pET32a/Os4BGlu18 insoluble protein; Lane 17, 0.8 mM IPTG pET32a/Os4BGlu18 culture soluble protein; Lane 18, 0.8 mM IPTG pET32a/Os4BGlu18 culture insoluble protein.

3.4. Purification of Os4BGlu16

The N-terminally His_8-tagged Os4BGlu16 protein in *P. pastoris* was purified from the pichia media by IMAC. β-Glucosidase activity was detected in the media, as shown in Figure 3.2, and a broad protein band was detected above 67 kDa on the SDS-PAGE gel (Figure 3.13). To test whether the broadness of the protein band was due to glycosylation, deglycosylation with endoglycosidase H was done and a single band of approximately 60 kDa was detected on a Coomassie-stained SDS-PAGE gel (Figure 3.6).

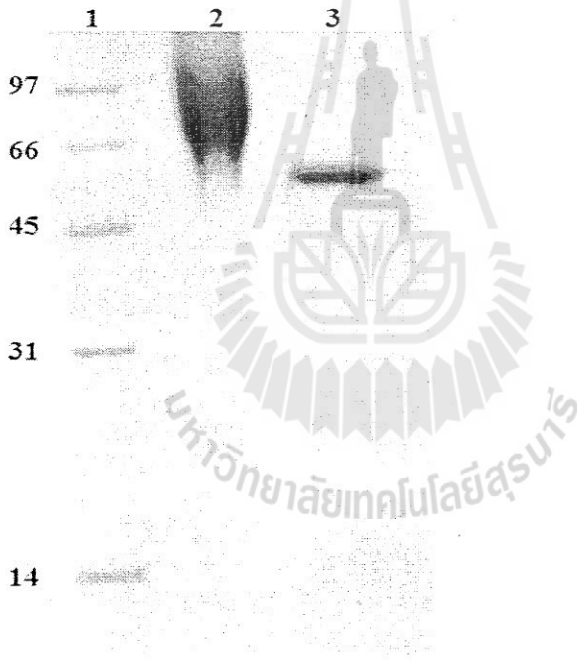


Figure 3.6 SDS-PAGE analysis of Os4BGlu16 protein expressed in *Pichia pastoris*.

Lane 1, standard protein marker; Lane 2, Os4BGlu16 protein before deglycosylation; Lane 3, Os4BGlu16 protein after deglycosylation with endoglycosidase H.

3.5. Effect of pH and temperature on the activity and stability of Os4BGlu16

As shown in Figure 3.7, the optimum pH for glycosylated Os4BGlu16 was found to be 6.5 and after deglycosylation the optimum pH for Os4BGlu16 was 6.0, but at pH 5.5 and 6.5 the activity was within error of this (only 4% and 2% lower, respectively). Figure 3.8 shows that the activity of Os4BGlu16 maintained >50% maximal activity when incubated up to an hour at pH values ranging from pH 5.5 to 11.5 at 25 °C, indicating that this enzyme was stable at neutral to basic pH, but lost its activity in the highly acidic range. The temperature optimum for both the glycosylated and deglycosylated forms of the enzyme was 45 °C (Figure 3.9), but it was thermostable over the range of 20-40 °C from 15-60 min and began to lose activity at 50 °C, while, at 60 °C no activity was detectable (Figure 3.10).

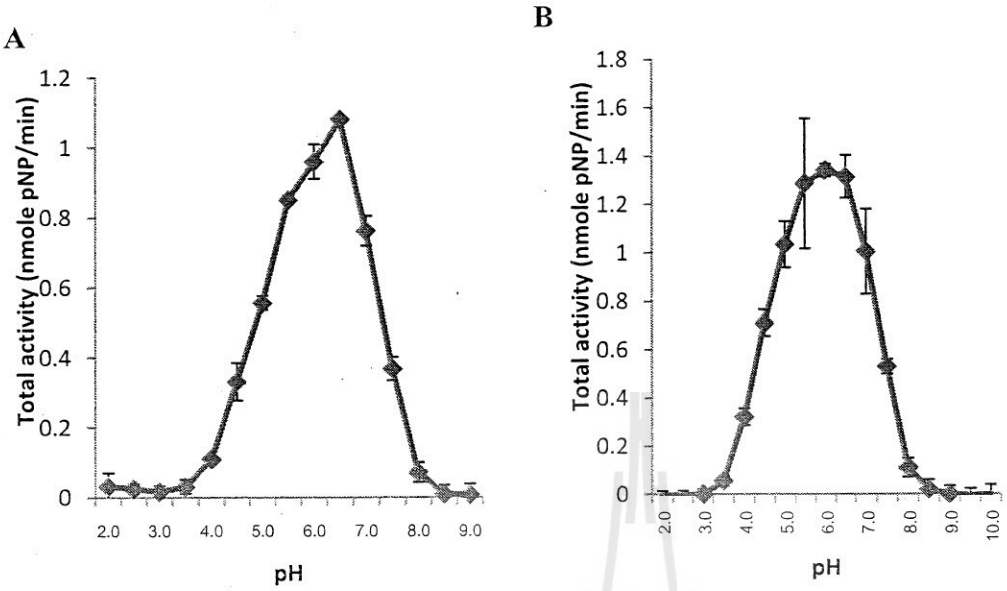


Figure 3.7 Activity versus pH profiles of Os4BGlu16. The purified enzyme was incubated with 1 mM *p*NPGlc in 100 mM McIlvaine's universal buffers (citric acid-disodium hydrogen phosphate) ranging from pH 2.0 to 9.0 at 30 °C for 30 min. A) pH optimum of recombinant Os4BGlu16 before deglycosylation; B) pH optimum of recombinant Os4BGlu16 after deglycosylation.

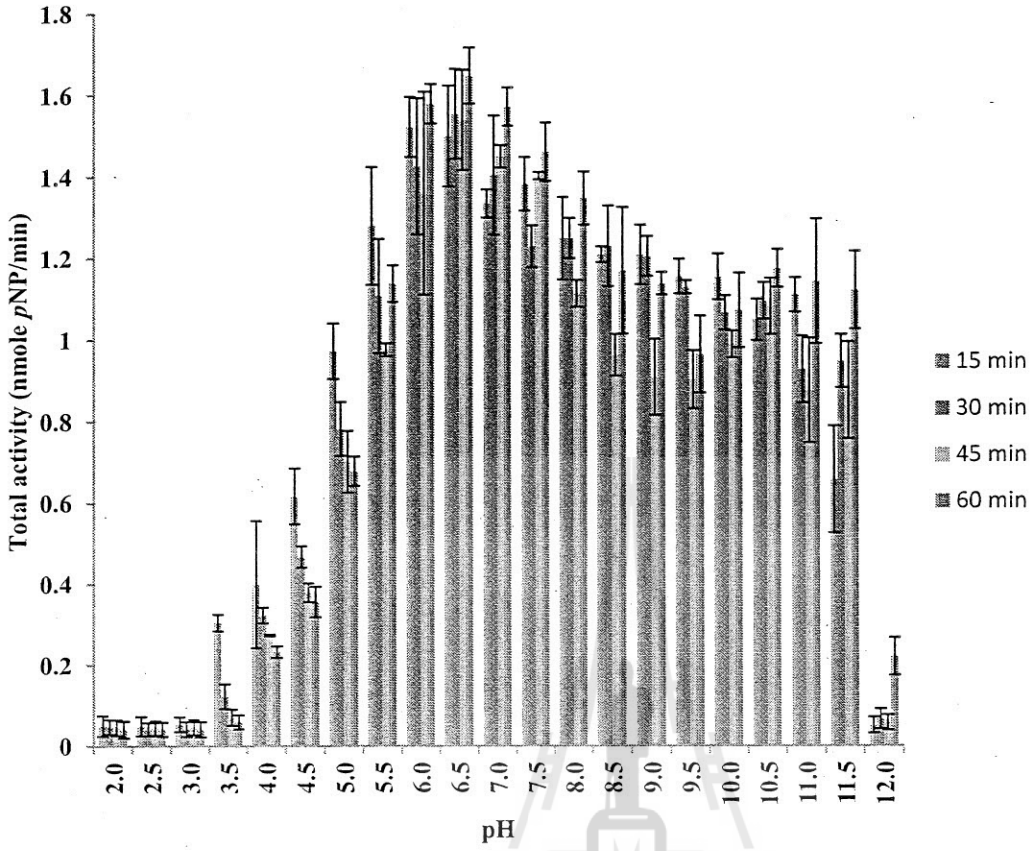


Figure 3.8 pH stability of Os4BGlu16 activity. The Os4BGlu16 was incubated in universal buffer (pH 2-12) for time periods of 15, 30, 45, and 60 min at 25 °C, then enzyme was diluted 100 fold into 100 mM sodium phosphate buffer, pH 6.5, and assayed with 1 mM *p*NPGlc for 30 min at 30 °C.

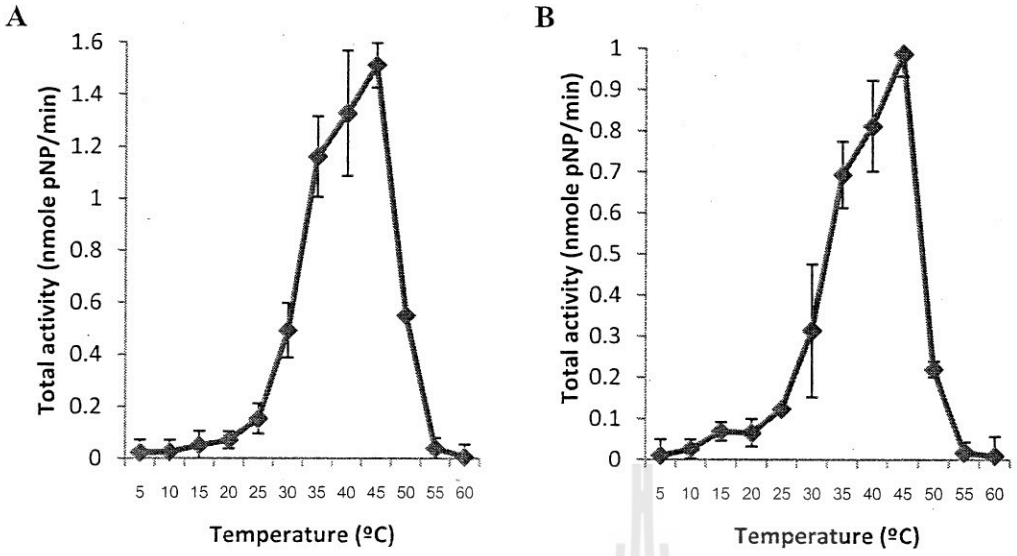


Figure 3.9 Temperature optima of Os4BGlu16. The activity was assayed by pre-incubating 1 μg of enzyme in 50 mM sodium phosphate buffer, pH 6.5, at the specified temperatures for 10 min to bring the reactions up to temperature, then the enzymes were assayed with 1 mM *p*NPGlc for 10 min at the same temperature as the preincubation. Panel A depicts the temperature optimum of recombinant Os4BGlu16 before deglycosylation while panel B shows the temperature optimum of recombinant Os4BGlu16 after deglycosylation.

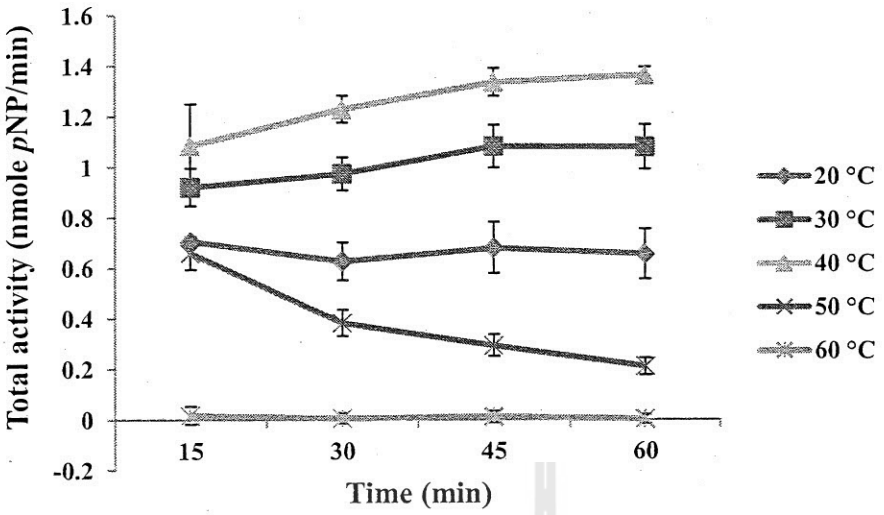


Figure 3.10 Thermostability of Os4BGlu16. The Os4BGlu6 was incubated at various temperatures (20-60 °C) from 15 to 60 min in 50 mM sodium phosphate buffer, pH 6.5, and enzyme activity was determined at 30 °C by adding 1 mM final concentration of *p*NPGlc and incubating for 30 min. Pre means preincubation time at 0 min.

3.6. Purification of Os4BGlu18

The Os4BGlu18 fusion protein with N-terminal thioredoxin, His_6 and S-tags was highly expressed in *E. coli* strain Origami(DE3) and induced with 0.1 mM IPTG at 18 °C for 16-18 h. The soluble protein of recombinant Os4BGlu18 was purified by anion-exchange chromatography, and the active protein started to elute at 0.3-0.5 M NaCl. Then, the active protein was loaded on hydrophobic interaction chromatography and eluted at 0.3-0.5 M NaCl concentration. Finally, the protein was purified by IMAC column as described in the Methods. Os4BGlu18 was produced at approximately 75 kDa and approximately 90% pure, as judged by SDS-PAGE (Figure 3.11).

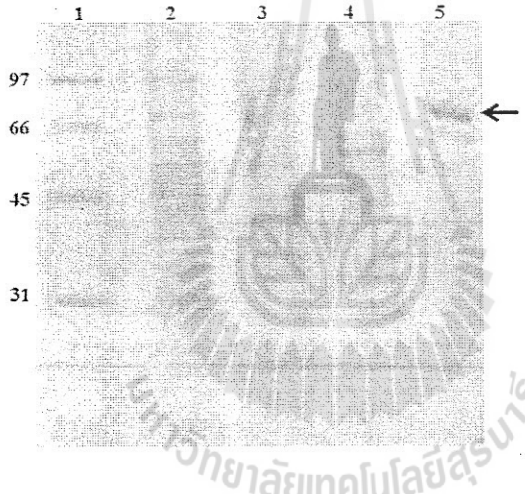


Figure 3.11 SDS-PAGE analysis of Os4BGlu18 production in *Escherichia coli*.

Lane 1, standard protein marker; Lane 2, crude protein extract of cells expressing Os4BGlu18; Lane 3, Os4BGlu18 purified by Q-sepharose ion exchange chromatography; Lane 4, Os4BGlu18 after purification by phenyl sepharose chromatography; Lane 5, Os4BGlu18 after purification by IMAC.

3.7. Effect of pH and temperature on the activity and stability of Os4BGlu18

The optimum pH for Os4BGlu18 was found to be 5.0 and the activity dropped by 40% and 60% at pH 3.5 and 6.5, respectively (Figure 3.12). Os4BGlu18 maintained similar activity when it was incubated 15-60 min at pH values from pH 4.0 to 8.0 at 25 °C (Figure 3.13), indicating that this enzyme was stable from pH 4.0 to 8.0, but unstable at pH ≤3.5 and ≥8.5. The temperature optimum for this enzyme is 55 °C (Figure 3.14), but it was only thermostable for 60 min between 20-40 °C and began to lose activity at 50 °C in 30 min (Figure 3.15).

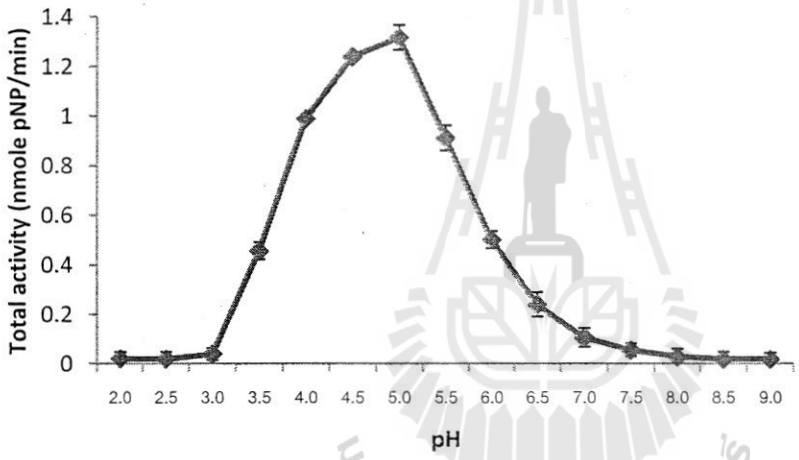


Figure 3.12 Activity versus pH profile of Os4BGlu18 purified from *E. coli*. The purified enzyme was incubated with 1 mM *p*NPGlc in 100 mM McIlvaine's universal buffers (citric acid-disodium hydrogen phosphate) ranging from pH 2.0 to 9.0 at 30 °C for 30 min.

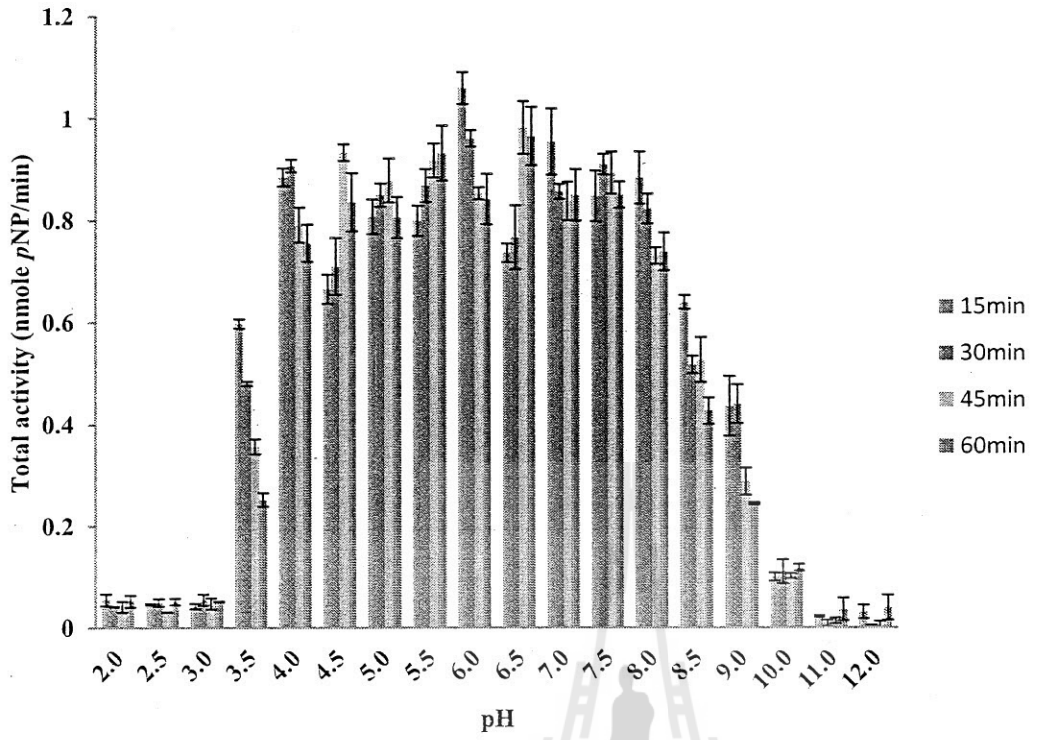


Figure 3.13 pH stability of Os4BGlu18 activity. The Os4BGlu18 was incubated in universal buffer (pH 2-12) for time periods of 15, 30, 45, and 60 min at 25 °C, then was diluted 100 fold into 100 mM sodium acetate buffer, pH 5.0, and assayed with 1 mM *p*NPGlc for 30 min at 30 °C.

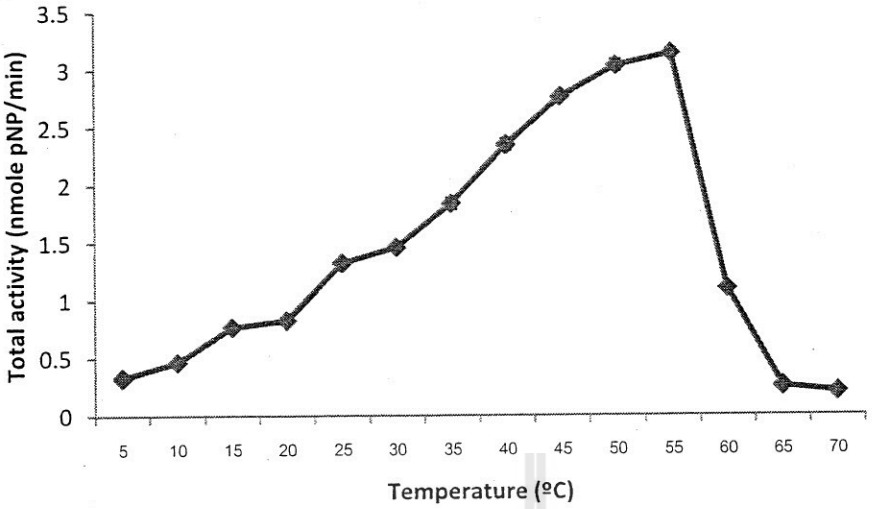


Figure 3.14 Temperature optimum of Os4BGlu18 fusion protein expressed in *E. coli*. The activity was assayed by pre-incubating 1 μg of enzyme in 50 mM buffer (sodium phosphate, pH 6.5, for Os4BGlu16 or sodium acetate, pH 5.0, for Os4BGlu18) at the specified temperatures for 10 min to bring the reactions up to temperature, then the enzymes were assayed with 1 mM *p*NPGlc for 10 min at the same temperature as the preincubation.

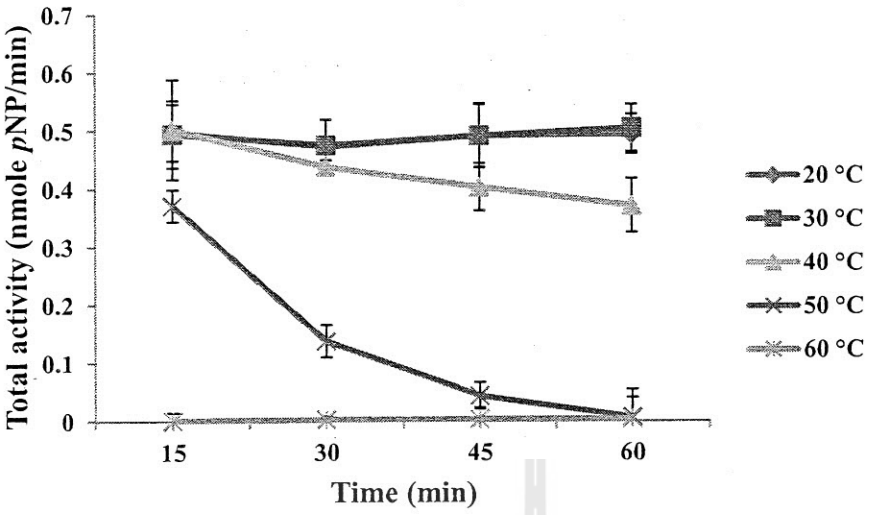


Figure 3.15 Thermostability of Os4BGlu18. The Os4BGlu18 was incubated at various temperatures (20-60 °C) from 15 to 60 min in 50 mM sodium acetate buffer, pH 5.0, and enzyme activity was determined at 30 °C by adding 1 mM final concentration of *p*NPGlc and incubating for 30 min. Pre means preincubation time at 0 min.

3.8. Substrate specificity and kinetic analysis of Os4BGlu16 and Os4BGlu18

The substrate specificities of the purified recombinant Os4BGlu16 and Os4BGlu18 enzymes were determined by testing different *p*NP glycosides and natural and synthetic glucosides, as shown in Table 3.1. First, the glycone specificities of both enzymes were investigated by assaying their activities toward β- and α-linked *p*-nitrophenyl (*p*NP)-glycosides. Among all the NP glycosides tested, Os4BGlu16 hydrolyzed *p*NP-β-D-fucopyranoside best, followed by *p*NPGlc, *o*NP-β-D-glucopyranoside, *p*NP-β-D-galactopyranoside and *p*NP-β-D-xylopyranoside, respectively. Among the NP-glycosides, Os4BGlu18 hydrolyzed the same substrates as Os4BGlu16. Os4BGlu16 hydrolyzed *o*NP-β-D-glucopyranoside with higher activity than *p*NP-β-D-galactopyranoside, while Os4BGlu18 displayed low activity toward *o*NP-β-D-glucopyranoside, suggesting a difference in aglycone specificity. To evaluate further their aglycone specificities, hydrolysis of natural and synthetic glycosides and oligosaccharides was tested by TLC analysis. Os4BGlu16 and Os4BGlu18 could hydrolyze the monolignol glucosides *p*-coumarol β-D-glucoside, coniferin and syringin, along with daidzin, esculin, helicin, salicin, indoxyl β-D-glucoside, 4-methylumbelliferyl β-D-glucoside and 4-methylumbelliferyl β-D-fucoside. Moreover, Os4BGlu18 also hydrolyzed arbutin, methyl β-D-glucoside, *n*-octyl β-D-glucoside and *n*-heptyl β-D-glucoside. Neither enzyme could hydrolyze β-1,3-, β-1,4-, or β-1,6-linked gluco-oligosaccharides.

Table 3.1 Relative activities of Os4BGlu16 and Os4BGlu18 toward nitrophenyl glycosides.

Substrates	Relative activity (%)*	
	Os4BGlu16	**Os4BGlu18**
***p*NP glycosides**		
*p*NP-β-D-glucopyranoside	100.0±1.9	100.0±4.8
*p*NP-β-D-mannopyranoside	NA	NA
*p*NP-β-D-fucopyranoside	297.3±5.9	214.2±2.8
*p*NP-β-D-galactopyranoside	31.8±2.5	38.4±3.9
*p*NP-β-D-maltopyranoside	NA	NA
*p*NP-β-D-cellobiopyranoside	NA	NA
*p*NP-β-D-xylopyranoside	25.8±0.4	23.1±0.8
*p*NP-1-thio-β-D-glucopyranoside	NA	NA
*p*NP-β-L-arabinopyranoside	NA	NA
*o*NP-β-D-glucopyranoside	46.2±2.5	3.7±1.6
*p*NP-α-D-glucopyranoside	NA	NA
*p*NP-α-D-mannopyranoside	NA	NA
*p*NP-α-D-galactopyranoside	NA	NA
*p*NP-α-D-glucopyranoside	NA	NA
*p*NP-α-L-fucopyranoside	NA	NA
*p*NP-N-acetyl-β-D-glucosaminide	NA	NA

*The substrates were assayed at 1 mM substrate concentrations at 30 °C for 30 min.

**NA means no activity detected.

Table 3.2 Activities of Os4BGlu16 and Os4BGlu18 toward natural and synthetic glycosides based on TLC analysis.

Natural and synthetic glucosides	Activity	
	Os4BGlu16	**Os4BGlu18**
Monolignol glucosides		
p-Coumarol glucoside	+	+
Coniferin	+	+
Syringin	+	+
Natural glucosides		
D-Amygdalin	NA	NA
Quercetin-3-glucoside	NA	NA
Phlorizin	NA	NA
Daidzin	+	+
Gossypin	NA	NA
Mangiferin	NA	NA
Esculin	+	+
Arbutin	NA	+
Helicin	+	+
Salicin	+	+
Naringin	NA	NA
Dhurrin	NA	NA
Apigenin 7-glucoside	NA	NA

The "+" sign means activity detected, while "NA" means no activity detected.

Table 3.2 Activities of Os4BGlu16 and Os4BGlu18 toward natural and synthetic glycosides based on TLC analysis (Continued).

Substrates	Activity	
	Os4BGlu16	**Os4BGlu18**
Pyridoxyl β-D-glucoside	NA	NA
Trans-zeatin-glucoside	NA	NA
Indoxyl β-D-glucoside	+	+
Alkyl glycosides		
Methyl β-D-glucoside	NA	+
n-octyl β-D-glucoside	NA	+
n-heptyl β-D-glucoside	NA	+
4-methylumbelliferyl β-D-glucoside	+	+
4-methylumbelliferyl β-D-fucoside	+	+
Thioglycosides		
Octyl β-D-thio-glucoside	NA	NA
Isopropyl β-D-thiogalactoside	NA	NA
Oligosaccharides		
Sophorose	NA	NA
Laminari oligosaccharides	NA	NA
Cello oligosaccharides	NA	NA
Gentiobiose	NA	NA

The "+" sign means activity detected, while "NA" means no activity detected.

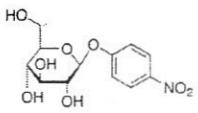
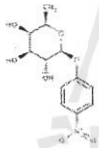
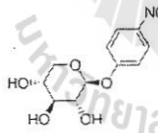
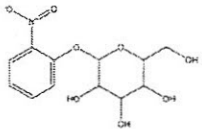
The kinetic parameters for hydrolysis of *p*NP-β-D-glucopyranoside, *p*NP-β-D-fucopyranoside, *p*NP-β-D-xylopyranoside, *o*NP-β-D-glucopyranoside, *p*-coumarol β-D glucoside, coniferin, syringin, salicin, helicin, *n*-octyl β-D-glucoside, *n*-heptyl β-D-glucoside and 4-methylumbelliferyl β-D-glucoside were determined for Os4BGlu16 and Os4BGlu18 (Table 3.3). Among *p*NP-glycosides, Os4BGlu16 has highest catalytic efficiency with *p*NPGlc (k_{cat}/K_M=1.062), followed by *p*NP-β-D-fucopyranoside (k_{cat}/K_M=0.896), *o*NP-β-D-glucopyranoside (k_{cat}/K_M=0.275) and *p*NP-β-D-xylopyranoside (k_{cat}/K_M=0.263). Os4BGlu18 preferred *p*NP-β-D-fucopyranoside (k_{cat}/K_M=4.06), followed by pNPGlc (k_{cat}/K_M=0.789) and *p*NP-β-D-xylopyranoside (k_{cat}/K_M=0.302) (Table 3.3).

Among monolignol glucoside substrates, Os4BGlu16 hydrolyzed syringin with a k_{cat}/K_M of 22.8 $mM^{-1}s^{-1}$, followed by coniferin and *p*-coumarol glucoside, with k_{cat}/K_M values of 21.6 and 6.2 $mM^{-1}s^{-1}$, respectively. In comparison, Os4BGlu18 hydrolyzed coniferin with a k_{cat}/K_M of 31.9 $mM^{-1}s^{-1}$, followed by syringin and *p*-coumarol glucoside, with k_{cat}/K_M values of 24.0 and 1.41 $mM^{-1}s^{-1}$, respectively. However, these high k_{cat}/K_M values were driven by the high k_{cat} values, with rather high K_M for these substrates, and both enzymes have lower K_M values for syringin than for the other two monolignol glucosides (Table 3.4). The high K_M and k_{cat}/K_M values for these substrates would make the rates of monolignol glucoside hydrolysis by Os4BGlu16 and Os4BGlu18 highly sensitive to the substrate concentrations in the plant.

Hydrolysis of other synthetic and natural glycosides was tested to assess the aglycone specificity of Os4BGlu16 and Os4BGlu18. Os4BGlu16 hydrolyzed helicin, 4-methylumbelliferyl β-D-glucopyranoside, and salicin with k_{cat}/K_M values of 0.145, 0.139, and 0.035 $mM^{-1}s^{-1}$, respectively. Os4BGlu18 showed broader substrate specificity than that of Os4BGlu16, in that it could hydrolyze 4-methylumbelliferyl β-

D-glucopyranoside, *n*-octyl β-D-glucopyranoside, *n*-heptyl β-D-glucopyranoside, and salicin with k_{cat}/K_M values of 1.054, 0.998, 0.452, and 0.0321 $mM^{-1}s^{-1}$, respectively (Table 3.4).

Table 3.3 Kinetic parameters of Os4BGlu16 and Os4BGlu18 for hydrolysis of nitrophenyl glycosides.

Substrate	Chemical structure	Os4BGlu16	Os4BGlu18
***p*NP-β-D-glucopyranoside**			
K_M (mM)		2.88±0.26	4.84±0.15
k_{cat} (s^{-1})		3.05±0.22	3.82±0.16
k_{cat}/K_M ($mM^{-1}s^{-1}$)		1.062±0.077	0.789±0.012
***p*NP-β-D-fucopyranoside**			
K_M (mM)		7.72±0.45	5.86±0.58
k_{cat} (s^{-1})		6.92±0.15	23.8±2.3
k_{cat}/K_M ($mM^{-1}s^{-1}$)		0.896±0.035	4.06±0.07
***p*NP-β-D-xylopyranoside**			
K_M (mM)		2.99±0.27	1.26±0.064
k_{cat} (s^{-1})		0.788±0.028	0.381±0.009
k_{cat}/K_M ($mM^{-1}s^{-1}$)		0.263±0.034	0.302±0.018
***o*NP-β-D-glucopyranoside**			
K_M (mM)		23.1±1.1	ND
k_{cat} (s^{-1})		6.34±0.52	ND
k_{cat}/K_M ($mM^{-1}s^{-1}$)		0.275±0.014	ND

ND means not determined due to low activity.

Table 3.4 Kinetic parameters of Os4BGlu16 and Os4BGlu18 for hydrolysis of monolignol glucosides and other aryl and alkyl glucosides.

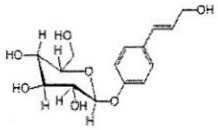
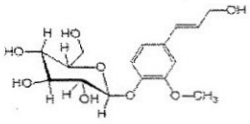
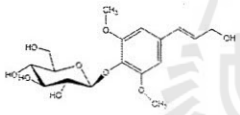
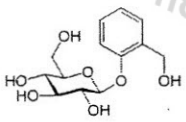
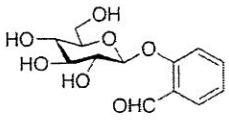
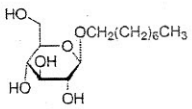
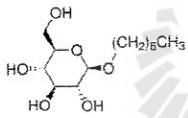
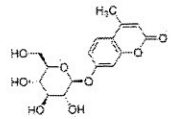
Substrate	Chemical structure	Os4BGlu16	Os4BGlu18
***p*-coumarol glucoside**			
K_M (mM)		13.10±0.88	8.15±0.20
k_{cat} (s^{-1})		81.5±3.0	11.49±0.08
k_{cat}/K_M ($mM^{-1}s^{-1}$)		6.22±0.19	1.411±0.024
coniferin			
K_M (mM)		19.9±2.2	8.02±0.19
k_{cat} (s^{-1})		429±31	255.8±4.1
k_{cat}/K_M ($mM^{-1}s^{-1}$)		21.6±0.89	31.9±0.3
syringin			
K_M (mM)		4.66±0.13	5.34±0.06
k_{cat} (s^{-1})		106.3±2.1	127.9±1.7
k_{cat}/K_M ($mM^{-1}s^{-1}$)		22.8±0.2	24.0±0.1
salicin			
K_M (mM)		0.622±0.058	5.41±0.21
k_{cat} (s^{-1})		0.0218±0.0027	0.1736±0.0009
k_{cat}/K_M ($mM^{-1}s^{-1}$)		0.0350±0.0010	0.0321±0.0011

Table 3.4 Kinetic parameters of Os4BGlu16 and Os4BGlu18 for hydrolysis of monolignol glucosides and other aryl and alkyl glucosides (Continued).

Substrate	Chemical structure	Os4BGlu16	Os4BGlu18
Helicin			
K_M (mM)		18.4±1.4	ND
k_{cat} (s^{-1})		2.66±0.12	ND
k_{cat}/K_M ($mM^{-1}s^{-1}$)		0.145±0.005	ND
***n*-octyl β-D-glucopyranoside**			
K_M (mM)		ND	8.05±0.64
k_{cat} (s^{-1})		ND	8.03±0.15
k_{cat}/K_M ($mM^{-1}s^{-1}$)		ND	0.998±0.067
***n*-heptyl β-D-glucopyranoside**			
K_M (mM)		ND	24.5±2.0
k_{cat} (s^{-1})		ND	11.05±0.66
k_{cat}/K_M ($mM^{-1}s^{-1}$)		ND	0.452±0.010
4-methyl umbelliferyl β-D-glucopyranoside			
K_M (mM)		4.62±0.38	9.39±0.48
k_{cat} (s^{-1})		0.641±0.027	9.89±0.22
k_{cat}/K_M ($mM^{-1}s^{-1}$)		0.139±0.005	1.054±0.049

ND means not determined, due to low activity.

3.9.*In planta* expression analysis

Quantitative real-time RT-PCR was used to measure the level of the putative monolignol β-glucosidases gene expression in various rice organs. The Os4BGlu14 gene is most highly expressed in reproductive tissues, especially in endosperm, lemma, embryo, pollen, panicle and flower (Figure 3.16). Os4BGlu16 is most highly expressed in 4 weeks to 10 weeks-old leaves, endosperm and lemma (Figure 3.17). Os4BGlu18 is most highly expressed in 1-week-old seedling, stem, leaf and leaf sheath at 4 weeks, pollen and lemma, and moderately expressed in other young vegetative tissues (Figure 3.18).

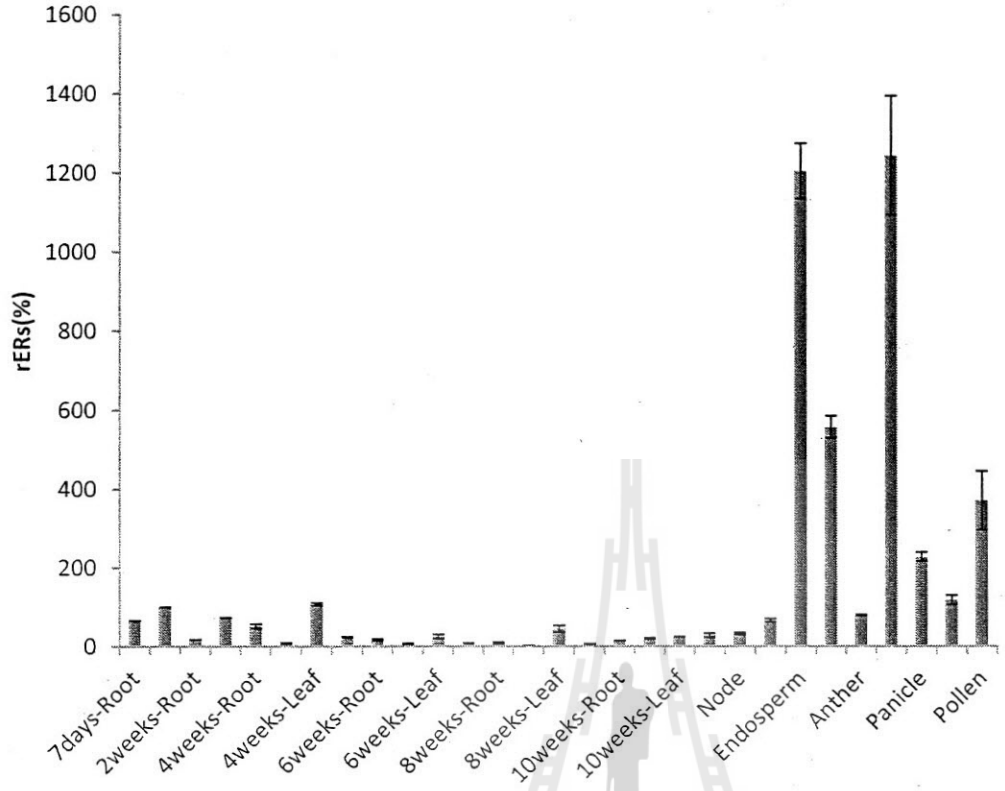


Figure 3.16 Gene expression analysis of rice Os4BGlu14 from qRT-PCR. RNA was extracted from tissues from 7 days to 10 weeks and endosperm and embryo of mature, dried seed, and the relative mRNA amounts for each gene assessed by qRT-PCR with gene-specific primers. Actin was used as a reference gene and 7-day shoots as the reference tissue, which is 100% in the graphs.

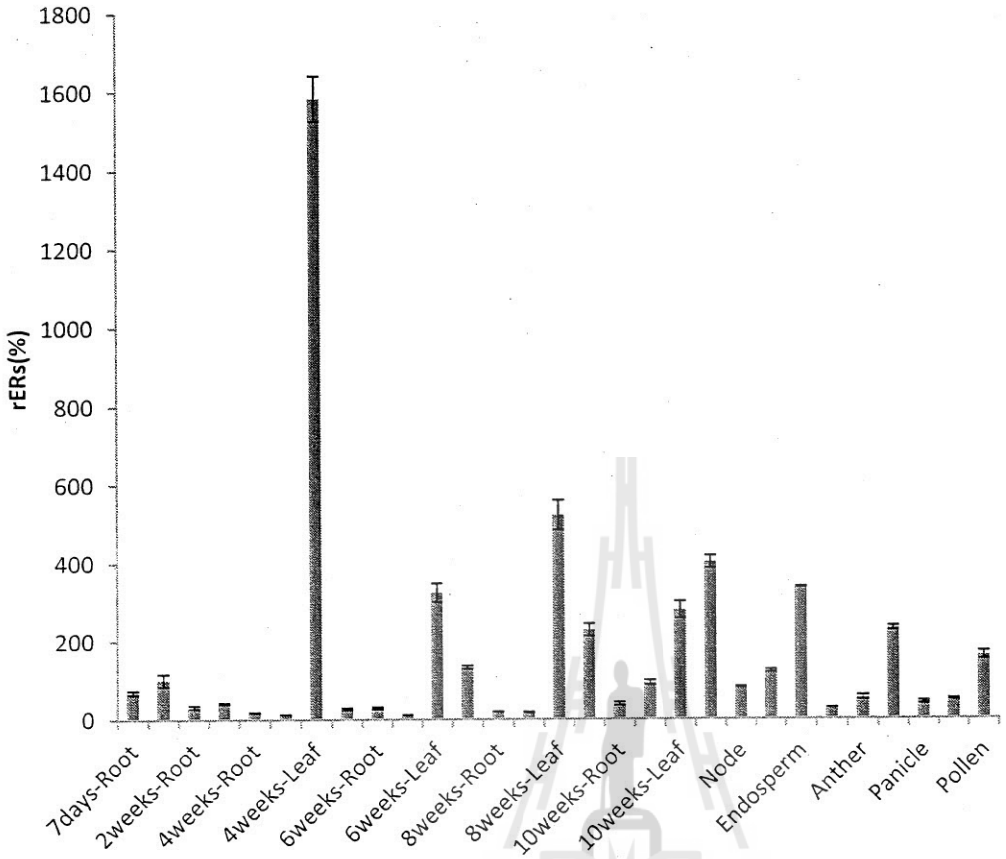


Figure 3.17 Gene expression analysis of rice Os4BGlu16 from qRT-PCR. RNA was extracted from tissues from 7 days to 10 weeks and endosperm and embryo of mature, dried seed, and the relative mRNA amounts for each gene assessed by qRT-PCR with gene-specific primers. Actin was used as a reference gene and 7-day shoots as the reference tissue, which is 100% in the graphs.

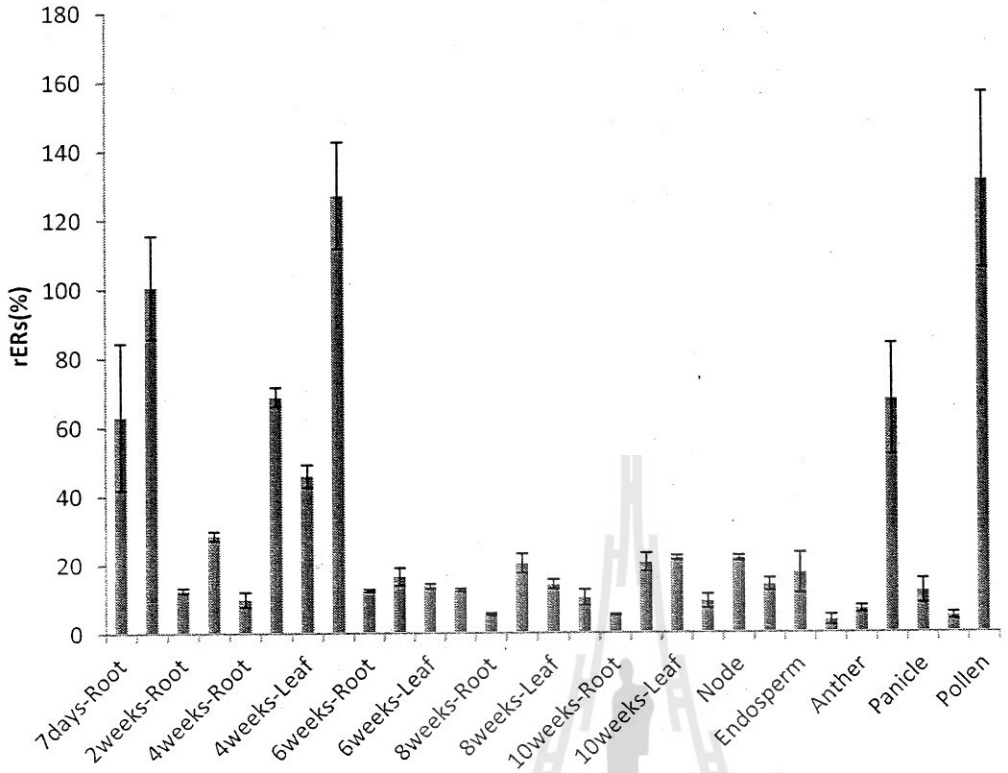


Figure 3.18 Gene expression analysis of rice Os4BGlu18 from qRT-PCR. RNA was extracted from tissues from 7 days to 10 weeks and endosperm and embryo of mature, dried seed, and the relative mRNA amounts for each gene assessed by qRT-PCR with gene-specific primers. Actin was used as a reference gene and 7-day shoots as the reference tissue, which is 100% in the graphs.

บทที่ 4

สรุปผลการวิจัย

The amino acid sequences of the putative rice β-glucosidases Os4BGlu14, Os4BGlu16, and Os4BGlu18 are closely related to characterized monolignol β-glucosidases, including *Arabidopsis* BGLU45 and BGLU46 and *Pinus contorta* coniferin β-glucosidase. The cDNA sequence of Os4BGlu14 encodes 516 amino acids, with the N-terminal 23 residues predicted to be a secretory signal peptide and the remainder a mature protein of 493 residues. However, the catalytic acid/base glutamate residue of Os4BGlu14 is replaced by glutamine, which led to the prediction that it may be an inactive β-glucosidase. The cDNA sequence of Os4BGlu16 also encodes 516 amino acids, including a predicted N-terminal secretory signal peptide of 27 residues and mature protein of 489 residues. Similarly, the cDNA sequence of Os4BGlu18 encodes a polypeptide of 505 amino acids, which were predicted to include an N-terminal secretory signal peptide of 26 residues and a mature protein of 479 residues.

The gene optimized for Os4BGlu16 expression in *P. pastoris* was inserted in the recombinant pPICZαB(NH_8)/Os4BGlu16 plasmid, which was used to express Os4BGlu16 as a secreted protein from *P. pastoris* strain SMD1168. Os4BGlu16 enzyme was successfully purified to produce an apparently homogeneous protein with only one IMAC step from the *Pichia* media.

The gene encoding mature Os4BGlu18 that was cloned into the pET32a expression vector was used to express a thioredoxin-Os4BGlu18 fusion protein in *E. coli* Origami(DE3) at 18 °C for 16-18 h. The catalytically active Os4BGlu18 enzyme was purified to get a single prominent protein with ion-exchange chromatography, hydrophobic interaction chromatography and IMAC, respectively.

The optimum pH of Os4BGlu16 was found to be 6.0-6.5 with deglycosylation having a negligible effect, but its activity was stable after incubation from pH 5.5 to 11.5 over a period of 15 min up to 60 min at 25 °C. The temperature optimum for both the glycosylated and deglycosylated forms of the Os4BGlu16 enzyme is 45 °C, but it was thermostable only over the range of 20-40 °C from 15-60 min. Meanwhile, the optimum pH for Os4BGlu18 was found to be 5.0. The activity of Os4BGlu18 maintained similar activity when incubated 15-60 min at pH values from pH 4.0 to 8.0 at 25 °C. The temperature optimum for Os4BGlu18 enzyme is 55 °C, but it was only thermostable over the range of 20-40 °C from 15-60 min.

Among *p*NP glycosides, Os4BGlu16 and Os4BGlu18 hydrolyzed *p*NP-β-D-fucopyranoside, *p*NP-β-D-glucopyranoside (*p*NPGlc), *o*NP-β-D-glucopyranoside, *p*NP-β-D-galactopyranoside and *p*NP-β-D-xylopyranoside, respectively, in overall order of activity, suggesting they have similar sugar preference. For hydrolysis of natural and synthetic glycosides and oligosaccharides substrates, Os4BGlu16 and Os4BGlu18 could hydrolyze the monolignol glucosides *p*-coumarol β-D-glucoside, coniferin and syringin, along with daidzin, esculin, helicin, salicin, indoxyl β-D-glucoside, 4-methylumbelliferyl β-D-glucoside and 4-methylumbelliferyl β-D-fucoside. In addition, Os4BGlu18 hydrolyzed arbutin, methyl β-D-glucoside, *n*-octyl β-D-glucoside and *n*-heptyl β-D-glucoside. Neither enzyme could hydrolyze β-1,3-, β-1,4-, or β-1,6-linked gluco-oligosaccharides, which suggests neither enzyme plays a role in cell wall degradation.

Os4BGlu16 and Os4BGlu18 have more efficiency to hydrolyze monolignol glucoside substrates than other natural and synthetic substrates. Os4BGlu16 has high catalytic efficiency toward syringin (k_{cat}/K_M of 22.8 $mM^{-1}s^{-1}$), coniferin (k_{cat}/K_M 21.6

$mM^{-1}s^{-1}$) and *p*-coumarol glucoside (k_{cat}/K_M 6.2 $mM^{-1}s^{-1}$). In comparison, Os4BGlu18 hydrolyzed coniferin best (k_{cat}/K_M 31.9 $mM^{-1}s^{-1}$), followed by syringin (k_{cat}/K_M 24.0 $mM^{-1}s^{-1}$) and *p*-coumarol glucoside (k_{cat}/K_M 1.41 $mM^{-1}s^{-1}$), respectively. These high k_{cat}/K_M values were driven by the high k_{cat} values, since the K_M values were rather high. This study supports the hypothesis that Os4BGlu16 and Os4BGlu18 are monolignol β-glucosidase enzymes and suggests the enzymes would be quite sensitive to changes in substrate levels in the plant.

Analysis of the gene expression level of the putative monolignol β-glucosidases in various rice organs showed that the *Os4BGlu14* gene is highly expressed in reproductive tissues, especially in endosperm, lemma, embryo, pollen, panicle and flower. *Os4BGlu16* is highly expressed in leaf from 4 weeks to 10 weeks, endosperm and lemma. *Os4BGlu18* is highly expressed in 1-week-old seedling, stem, leaf and leaf sheath at 4 weeks, pollen and lemma, and moderately expressed in other young vegetative tissues. This result suggested that Os4BGlu14 enzyme may primarily function in the reproductive tissue, while Os4BGlu16 and Os4BGlu18 apparently function in both vegetative and reproductive tissues.

In sumary, several lines of evidence support the idea that Os4Bglu16 and Os4Bglu18 are monolignol β-glucosidase that may act in the lignification system in rice plants. First, successful recombinant expression and purification allowed us to see that Os4BGlu16 and Os4BGlu18 enzymes have higher efficiency to hydrolyze monolignol glucoside substrates than other substrates. Second, the pH optimum of Os4BGlu16 and Os4BGlu18 is in the acidic range, consistent with a role in the apoplast or acidic vacuole. The synthesis of monolignols from phenylalanine and shikimate is carried out by cytosolic and ER membrane-anchored cytosolic enzymes. Monolignols and lignans may then be conjugated by UTP-glucose-dependent glucosyltransferases

and then transported to the vacuole or the monolignols may be directly transported to the cell wall for oxidative cross-linking by apoplastic peroxidases and laccases into lignins (Wang et al., 2013), so a role in further processing of monoglucosides in the vacuoles or upon release to the apoplast is possible for Os4BGlu16 and Os4BGlu18. Last, the Os4BGlu14, Os4BGlu16, and Os4BGlu18 expression was found to occur in the tissues that undergo lignification from vegetative to reproductive stage, and were in line with and the build-up of monolignol compounds in this study. Once we understand these roles better, this information may be used for improving rice resistance to disease by lignification for fungal resistance, or for producing low-lignin rice straw for biofuel production.

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   7.1 Journal Publications

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7.2. Projects as Head of Project (หัวหน้าโครงการวิจัย): ชื่อโครงการวิจัย...

7.2.1. Homology-Based Screening of Glycosidases from Thai Plants, 2539-2542, Thailand Research Fund Young Researcher Development Grant, Completed

7.2.2. Characterization of Glycosidases from Forest Legumes, 2542-2545, SUT/NRCT, Completed

7.2.3. Expression and Characterization of Thai Plant Glycosyl Hydrolases, 2545-2548, Completed

7.2.4. Investigation of Rice Beta-Glycosidase Gene Functions, 2546-2549, National Science and Technology Development Agency, Grant BT-B-06-RG-19-4608, Completed

7.2.5. Enzymatic Screening and Characterization of Thai Plant Glycosides, 2547-2550, SUT/NRCT, Completed

7.2.6. Structure and Function Relationships in Plant Beta-Glucosidases, 2547-2550, Thailand Research Fund Basic Research Grant BRG4780024, Completed

7.2.7. Structural Studies of Carbohydrate Active Enzymes from Rice, 2549-2552, National Synchrotron Research Center, Completed.

7.2.8. Structure and Function Relationships in Plant Beta-Glucosidases II, 2550-2553, Thailand Research Fund Basic Research Grant BRG5080007, Completed 10/2553.

7.2.9. Structural Basis for Substrate-Specificity in Glucooligosaccharide Hydrolyzing β-Glucosidases from Glycosyl Hydrolase Families 1 and 3, 2553-2556, Thailand Research Fund Basic Research Grant BRG53_0017.

7.2.10. Characterization of a glycoside hydrolase family 1 group 6 hydrolase, 2554. SUT/NRCT.

7.2.11. Structure and Function Relationships in Plant Beta-Glucosidases II, 2550-2553, Thailand Research Fund Basic Research Grant BRG5080007

7.2.12. Structural Basis for Substrate-Specificity in Glucooligosaccharide Hydrolyzing β-Glucosidases from Glycosyl Hydrolase Families 1 and 3, 2553-2556, Thailand Research Fund Basic Research Grant BRG53_0017.

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4. ตำแหน่งปัจจุบัน รองศาสตราจารย์

5. หน่วยงานที่อยู่ที่ติดต่อได้

สาขาวิชาเทคโนโลยีชีวภาพ สำนักวิชาเทคโนโลยีการเกษตร

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6. ประวัติการศึกษา

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7. สาขาวิชาการที่มีความชำนาญพิเศษ (แตกต่างจากวุฒิการศึกษา) ระบุสาขาวิชาการ

- Molecular Biology & Genetic Engineering (Plant & Animal)
- Recombinant Protein Production
- Bioinformatics

8. ประสบการณ์ที่เกี่ยวข้องกับการบริหารงานวิจัยทั้งภายในและภายนอกประเทศ : ระบุสถานภาพในการ

ทำการวิจัยว่าเป็นผู้อำนวยการแผนงานวิจัย หัวหน้าโครงการวิจัย หรือผู้ร่วมวิจัยในแต่ละข้อเสนอโครงการวิจัย เป็นต้น

ผู้อำนวยการแผนงานวิจัย : การผลิตกรดซักซินิกโดยเชื้อแบคทีเรีย (วช)

ผู้ประสานงานชุดโครงการ การวิจัยโปรตีนแห่ง มหาวิทยาลัยเทคโนโลยีสุรนารี

หัวหน้าโครงการวิจัย : มีชื่อโครงการวิจัย ดังต่อไปนี้

- โมโนโคลนัลแอนติบอดีต่ออสุจิ Y ของวัว
- การโคลนและผลิตโปรตีนไฟโคไซยานินจากไซยาโนแบคทีเรีย คาดว่าจะส่งรายงานการวิจัย ธันวาคม25 53
- การศึกษาคุณสมบัติของเอนไซม์เบต้ากลูโคซิเดสจากยีน SFR2 ในข้าว
- การพัฒนาเครื่องหมายการตรวจสอบย้อนกลับปลานิลจากมทส. ส่งรายงานการวิจัยฉบับสมบูรณ์ 2553
- การค้นหาและการแสดงออกของกลุ่มยีน Glycosyl Hydrolases ในจีโนมของข้าวหอมมะลิ คาดว่าจะส่งรายงานการวิจัย ธันวาคม 2553
- การโคลนและผลิตเอ็นไซม์เอนเทอโรไคเนสสายสั้น ส่งรายงานการวิจัยฉบับสมบูรณ์ 2550
- การผลิตรีคอมบิแนนท์ทรานสกลูตามิเสนจากปลานิล ส่งรายงานการวิจัยฉบับสมบูรณ์ 2550
- การผลิตรีคอมบิแนนท์เอนไซม์ Thermostable DNA polymerase จาก *Pyrococcus furiosus* ในแบคทีเรีย *Escherichia coli* ส่งรายงานการวิจัยฉบับสมบูรณ์25 50
- การศึกษาสภาวะที่เหมาะสมในการผลิตรีคอมบิแนนท์โปรตีนในถังหมัก ส่งรายงานการวิจัยฉบับสมบูรณ์254 9
- การแสดงออกและการผลิตเบต้ากลูโคสิเดส โดย *Pichia pastoris* ส่งรายงานการวิจัยฉบับสมบูรณ์ 2548
- การจำแนกลักษณะทางพันธุกรรม สรีระวิทยา และพฤติกรรมของไก่พื้นเมืองไทย

ส่งรายงานการวิจัยฉบับสมบูรณ์ 2547

- การหาแผนที่ทางพันธุกรรมของไผ่ตง ส่งรายงานการวิจัยฉบับสมบูรณ์ 2546

- การพัฒนาวิธีการตรวจหาชนิดของโครโมโซมเพศปลานิล ส่งรายงานการวิจัยฉบับสมบูรณ์ 2543
- การผลิตรีคอมบิแนนท์เอนไซม์ *Taq* DNA polymerase ส่งรายงานการวิจัยฉบับสมบูรณ์ 2541
- Investigation of Rice Beta-Glycosidase Gene Functions. (National Science and Technology Development Agency National Center for Genetic Engineering and Biotechnology) ส่งรายงานการวิจัยฉบับสมบูรณ์ 2550
- Clonal Selection of Sweet Bamboo for Commercial and Industrial Uses ส่ง รายงานการวิจัยฉบับสมบูรณ์ 2545
- Functional Analysis of the Maize bZIP Protein Opaque (NIH, USA) แล้วเสร็จ 2537

งานวิจัยที่ทำเสร็จแล้ว :

ดูหัวข้อก่อนหน้านี้

งานวิจัยที่กำลังทำ :

- โมโนโคลนัลแอนติบอดีต่ออสุจิ Y ของวัว 2553-2554 สถานภาพในการทำวิจัย : เริ่มโครงการในปีงบประมาณ 2553 และได้ดำเนินการไปแล้ว 30%
- การโคลนและผลิตโปรตีนไฟโคไซยานินจากไซยาโนแบคทีเรีย 2552-2553 สถานภาพในการทำวิจัย : เริ่มโครงการในปีงบประมาณ 2552 และได้ดำเนินการไปแล้ว 90% คาดว่าจะส่งรายงานการวิจัย ธันวาคม 2553
- การศึกษาคุณสมบัติของเอนไซม์เบต้ากลูโคซิเดสจากยีน SFR2 ในข้าว 2551-2552 สถานภาพในการทำวิจัย : เริ่มโครงการในปีงบประมาณ 2551 และได้ดำเนินการไปแล้ว 70%

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