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รายงานการวิจัย

การบ่งชี้และศึกษาการทำงานของจิบเบอริลินเบตากลูโคไซเดสจากข้าว

Identification and Characterization of Rice Gibberellin Beta-Glucosidase

ได้รับทุนอุดหนุนการวิจัยจาก

มหาวิทยาลัยเทคโนโลยีสุรนารี

ผลงานวิจัยเป็นความรับผิดชอบของหัวหน้าโครงการวิจัยแต่เพียงผู้เดียว

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คณะผู้วิจัย

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การวิจัยครั้งนี้ได้รับทุนอุดหนุนการวิจัยจาก มหาวิทยาลัยเทคโนโลยีสุรนารี ประจำปีงบประมาณ พ.ศ. 2555-2557 ผู้วิจัยขอขอบคุณมา ณ โอกาสนี้

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บทคัดย่อ

จิบเบอริลิน (gibberellins, GA) เป็นฮอร์โมนพืชที่มีบทบาทหลายอย่างในการเจริญเติบโตของพืช เช่น การเจริญเติบโตของต้นอ่อน การงอกของเมล็ด การสร้างเมล็ด และอื่นๆ ในพืช ฮอร์โมนจิบเบอริลิน จะถูกจับกับน้ำตาล และไม่ใช่น้ำตาลซึ่งจะทำให้ฮอร์โมนจิบเบอริลินไม่สามารถทำงานได้ ซึ่งคิดว่าเอนไซม์กลุ่มเอ็นไซม์กลูโคซิลทรานส์เฟอเรส (glycosyltransferases) ทำหน้าที่เติมกลูโคสทำให้จิบเบอริลินอยู่ในรูปกลูโคไซด์ (glucosides) และ ต่อกับน้ำตาลด้วยพันธะเอสเทอร์ (glucose esters) ได้มีการทดสอบพบว่าเอนไซม์ เบตา-กลูโคซิเดส (β-glucosidase) ทำหน้าที่ตัดกลูโคสออกจากจิบเบอริลินเพื่อให้จิบเบอริลินอยู่ในรูปทำงานได้ ในการศึกษานี้เราหาวิธีที่จะแยกและศึกษา gibberellin β-glucosidase จากข้าว ขั้นตอนแรกเราได้สังเคราะห์ gibberellin A4 glucose ester (GA_4-GE) เพื่อใช้ทดสอบกับเอนไซม์ เบตา-กลูโคซิเดส ที่ผลิตจากแบคทีเรีย และ ที่ได้จากต้นอ่อนข้าว ซึ่งได้ทำให้บริสุทธิ์ด้วยวิธีคอลัมน์โครมาโตกราฟี แล้วเลือกเฉพาะตัวอย่างมีเอนไซม์ หลังจากตกตะกอนโปรตีนด้วยแอมโมเนียมซัลเฟตแล้วผ่านการแยกให้บริสุทธิ์ อีก 6 ขั้นตอนพบว่ามีโปรตีน 2 ขนาด จากตัวอย่างที่สามารถย่อย GA_4-GE และได้ 0.18% ของกิจกรรมของเอนไซม์จากเดิมและความบริสุทธิ์เพิ่มขึ้น 337 เท่า ของเอนไซม์หลังจากตกตะกอนด้วยแอมโมเนียมซัลเฟต โปรตีนทั้ง 2 ขนาดถูกยืนยันโดยการย่อยโปรตีนที่ได้โดยการย่อยของเอนไซม์ทริปซิน เปปไทด์ที่ได้ทำการเคราะห์ด้วยเครื่อง mass spectrometer โดยหนึ่งในสอง ของโปรตีนนั้นพบเอนไซม์ไกลโคไซม์ ไฮโดรเลส ตระกูล family1 (glycoside hydrolase family 1 protein (GH1)) คือ Os4BGlu13 ที่สามารถย่อย GA_4-GE ได้ หรือมีชื่ออีกอย่างว่า tuberonic acid β-glucosidase. ในการตรวจหาเอนไซม์ที่สามารถย่อย GA_4-GE นั้นพบว่า Os3BGlu6 มีความจำเพาะต่อเอนไซม์สูง Os3BGlu6 ที่ถูกทำให้กลายพันธุ์ที่ตำแหน่งกรด-เบส และ นิวคลีโอไฟล์พบว่ามีความจำเพาะต่อ GA_4-GE ลดลง ซึ่งเป็นไปตามความคาดหมาย และนอกจากนั้นความจำเพาะต่อ *p*-nitrophenylglucoside (*p*NPGlc) และซับสเตรทอีกสองชนิดก็ให้ผลคล้ายกันเพื่อ glucosyl azides จากผลการทดลองพบว่าเอนไซม์สามารถตัด glucosyl ester โดยใช้กลไกและความต้องการคล้ายกัน จากการศึกษาทำให้เราเข้าใจ การทำงาน ของ gibberellin gluco-conjugates ในข้าว รวมไปถึงการตัดของ glucose-1-esters.

Abstract

Gibberellins are plant hormones that play roles in a number of processes, including shoot elongation, germination, seed formation and others. In plants, gibberellins are found in both free aglycone forms and as glycosides, which are lacking or attenuated in activity. While glucosyltransferases are thought to glucosylate the gibberellins to form glucosides and glucose esters, β-glucosidases have been shown to release the active aglycones from these glucoconjugates. Here, we sought to isolate and characterize a gibberellin β-glucosidase from rice. First gibberellin A4 glucose ester (GA_4-GE) was synthesized, then it was used to screen previously expressed recombinant rice β-glucosidases and to select fractions for the purification of rice β-glucosidase from seedling extract. After ammonium sulfate precipitation and 6 steps of chromatography, two bands of protein were left in the fraction which hydrolyzed GA_4-GE, and 0.18% of the activity was remaining at 337-fold purification from the crude precipitant solution. The two bands were identified by tryptic digestion and mass spectrometric analysis, and one of these was the glycoside hydrolase family 1 protein Os4BGlu13, also known as tuberonic acid β-glucosidase. This suggests that Os4BGlu13 can also serve as a gibberellin glucose ester β-glucosidase. In screening of recombinant enzymes for GA_4-GE hydrolysis, Os3BGlu6 was identified as an enzyme with relatively high activity toward the gibberellin substrate. Mutation of the catalytic acid/base and nucleophile of Os3BGlu6 showed that this had the expected decrease in activity with GA_4-GE substrate, as well as *p*-nitrophenylglucoside and the two substrates gave similar rescue with azide to produce the expected glucosyl azides. These results show that the enzyme hydrolyzes the glucosyl ester with a similar mechanism and requirements to its hydrolysis of glycosides. Overall, these results help us to understand the turnover of gibberellin gluco-conjugates in rice, as well as the hydrolytic hydrolysis of glucose-1-esters.

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Section 2
CHAPTER 1. Introduction

1.1. Importance and Background of Reseach Problem (ความสำคัญและที่มาของปัญหาการวิจัย)

1.1.1 Rice and Rice Gene Evaluation

Rice (*Oryza sativa* L.) is the most important food crop in the world, with the second being its fellow Grameaceae wheat (*Triticum aestivum* L.), while another grass, corn (*Zea mays* L.) serves as the most important feed source. Due to its small genome, rice became the first monocot to have its genome sequenced [International Rice Genome Project, 2005]. This, along with efforts to produce expressed sequence TAGS and full-length cDNA from rice RNA extracted from various tissues under various conditions has provided a wealth of information on the genes that are expressed and the sequences of their protein products [Kawasaki et al., 2001; Kikuchi et al., 2003]. Microarray experiments have also been done to assess gene expression, with much of the data obtained available in public data bases [Rensink and Buell, 2005]. Thus, a wealth of data is available from rice for investigation of important processes in plants, particularly monocots, which include the world's most important crops.

Aside from the sequence-related advantages for rice research, many experimental techniques have been generated for rice, including transformation with exogenous DNA via Agrobacteria, RNA interference-mediated gene knock-down and generation of gene-disrupted rice lines by activation of endogenous retrotransposons [Hirochika et al., 2001] or insertion of T-DNA [Jeon et al., 2000]. Rice gene insertion lines can be identified by a search of publicly available databases, and the functions of the disrupted genes can then be investigated by ordering the lines or by collaboration with the consortium or laboratory that generated the lines. Thus, there are many tools available for the investigation of rice gene functions.

1.1.2 Gibberellins

Gibberellins (GA) are a family of four-cycle diterpenoids, some of which act as critical phytohormones in plants [Buchanan et al., 2000]. Gibberellins are synthesized by plants, fungi and bacteria, but their effects are primarily found in plants. In plants, gibberellins play critical roles in seed germination, stem elongation, leaf expansion, flower opening and pollen development. Gibberellins were first isolated from the pathogenic fungus *Gibberella fujikuroi* from which their name is derived. The presence of large quantities of GAs as secondary metabolites in this fungus leads to the extensive overgrowth of infected rice plants, a phenomenum known as foolish seedling disease. Gibberellic acid (GA_3) was the first gibberellin to be structurally characterized, and is widely used to regulate plant growth and development. The pathway of gibberellin synthesis in the plant has been elucidated and many of the genes encoding the synthetic enzymes characterized. Gibberellin actions have been described to occur via a soluble receptor, which was first described in rice as a gene whose mutation led to a dwarf phenotype [Ueguchi-Tanaka et al., 2005, 2007]. Gibberellins are classified based on their structures as well as their function. Naturally occurring tetracyclic diterpenoid acids with structures based on the ent-gibberellane carbon skeleton (examples shown in Fig.1.1) are named GA_1 to GA_n in the order of their discovery (www. plant-hormone.info/gibberellins).

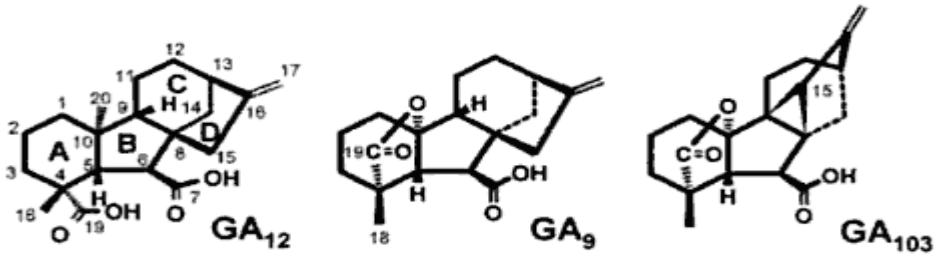

Figure 1.1 Examples of structures of gibberellins with different levels of structural complexity. GA_{12} is a C20-GA with the ent-gibberellane skeleton; GA_9 is the simplest C19-GA, which has an ent-20-norgibberellane skeleton, and GA_{103}, has an extra cyclopropane ring [Sponsel and Hedden, 2004].

Not all gibberellins exhibit bioactivity. Bioactive gibberellins exist in plants together with many inactive gibberellins and their glucosyl conjugates that may be inactive precursors or deactivation products of the active forms. The concentration of bioactive gibberellins in plants is in the range 10^{-11}-10^{-9} g/g fresh weight, depending on the tissue and species, and is closely regulated by the gibberellin biosynthetic and catabolic pathways [Sponsel and Hedden, 2004].

1.1.2.1. Gibberellin activities

Active gibberellins show many physiological effects, which depend on the type of gibberellin present, as well as the species of plant. Gibberellins are involved in many physiological processes [Davies, 1995; Mauseth, 1991; Raven, 1992; Salisbury and Ross, 1992]. They stimulate cell division and elongation, resulting in stem elongation. They stimulate bolting/flowering in response to day length. They break seed dormancy in some plants that require stratification or light to induce germination. They stimulate enzyme production (β-amylase) in germinating cereal grains, which allows mobilization of seed reserves. Gibberellins also induce maleness in dioecious flowers, can be used to cause parthenocarpic (seedless) fruit development and delay senescence in leaves and citrus fruits.

1.1.2.2. Gibberellin conjugates

Both as the free gibberellic acids and their conjugated forms exist in plants. The first GA-conjugate, GA_8-2-O-β-D-glucoside (GAs-2-O-G), was isolated and characterized from maturing fruits of *Phaseolus coccineus* [Schreiber et al., 1967, 1968, 1970]. Thereafter, a series of GA glucosyl conjugates have been isolated and structurally elucidated [Schreiber et al., 1976]. Beside these glucosyl conjugates, acyl [Schreiber et al., 1966] and alkyl [Hemphill et al., 1973] GA derivatives were also found in plants, although their biological significance is less understood. The glucosyl conjugation was found to be reversible in maize, as conjugated GA-glucosides produced from radioactive GA were found to be converted to free GA upon seed germination [Rood et al., 1983]. Currently, the conjugation process is considered to be an important aspect of GA metabolism in plants, although its physiological significance is unclear. Gibberellin conjugates may play an important role in the control of growth in higher plants in that they are potential metabolites of the GA biosynthetic pathways.

In addition to being the first GA conjugates isolated from plants, GA glucosyl conjugates, in which the GAs are connected to glucose, are also the most common. GA glucosyl conjugates can be divided into two groups: glucosyl ethers (or O-glucosides), where the glucose is linked to a hydroxy group of the GA skeleton, and glucosyl esters, in which the glucose is attached via the GA-C-7-carboxyl group. For those glucosyl conjugates that have been characterized so far, β-D-glucopyranose is found as the only structural form of the conjugating sugar moiety [Schneider and Schliemann, 1994]. For the GA-O-glucosides, the glucose moiety can be linked either to the 2-O-, 3-O-, 11-O-, 13-O- or 17-O- position of the parent GA [Sembdner et al., 1994]. In rice, the glucosyl ester of GA_1, GA_2, GA_4, GA_8 and GA_{34} have been found after application of [^{3}H] GA_4 to seedlings [Koshioka et al., 1988]. Although it has not been characterized to date, it is conceivable that this conjugation can affect either transport of the gibberellins into the cell or binding to their receptor in the cytoplasm or nucleus.

Conjugation of gibberellins to GA glucosyl conjugates causes loss of the biological activity, but the increased polarities of GA glucosyl conjugates likely favor GA conjugates being deposited into the vacuole. From the occurrence of gibberellin glucosyl conjugates in the bleeding sap of trees, it has been suggested that these conjugates may function in the long-distance gibberellin transport [Dathe et al., 1978, Dathe et al, 1982]. Because GA glucosyl conjugates form and accumulate during the seed maturation period, they have been proposed to function as storage products [Lenton and Appleford, 1991, Lenton et al., 1993]. However, this theory applies only to conjugates of biologically active GAs, where hydrolysis, for example during early stages of seed germination, releases free GAs prior to *de novo* GA biosynthesis.

1.1.2.3. Detection of gibberellins and their conjugates in plants

In order to understand the metabolism of gibberellins in the plants, we should know the concentrations of gibberellins as well as their conjugates in the plants. The active form of the hormone is important, but their metabolites and precursors are also important because they often give us the clues for understanding the regulation of hormone action and metabolism. Since the concentrations of gibberellins and their conjugates are very low in the plant, highly sensitive instruments are required. GC-MS, LC-MS and LC-MS/MS are usually used for the analysis.

Due to the broad range of polarities of GA conjugates, sample preparation is extremely difficult. It is difficult to analyze free GAs, GA-O-glucosides and GA glucosyl esters simultaneously, since no method can extract them all in high enough quantities for chromatographic analysis. The extraction method usually includes the first step to extract GA conjugates and GAs with methanol, and a second step to separate GA-glucosyl esters from GAs and GA-O-glucosides by ion exchange chromatography, for example by DEAE-Sephadex column [Schneider.et al., 1992, 1993]. The esterified GAs will be mainly found in the neutral fractions. All free GAs and GA-O-glucosides will be mainly found in the acidic fractions because of their carboxylic acid groups on the GA rings.

Further purification of these fractions depends on the final analytical method used and on the target compound of the analysis. Reverse phase high performance liquid chromatography (RP-HPLC) and derivatization are usually used for the continued purification. GAs and their glucosyl conjugates can be analyzed by GC-MS by preparing their trimethylsilyl (TMS) derivatives [Yokota et al., 1975]. However, the limited mass range of the average benchtop GC-MS (approx. 800 m/z) has a problem, because the molecular

weights of TMS derivatives of sugar conjugates are high and over the limit of the mass range. Thus, permethylation has been introduced for GA-O-glucoside analysis. This method can produce stable and smaller molecular size permethylated GA-O-glucosides, which are suitable for GC-MS analysis [Rivier et al., 1981]. The permethylation method also has other advantages in that the method can be used directly with the acidic fraction after DEAE-Sephadex chromatography and the corresponding derivatives of free GAs and GA-O-glucosides can be separated easily as groups by RP-HPLC. GC-MS has a good sensitivity for permethylated GA-O-glucosides. About 1 ng of compound is sufficient for a full scan spectrum with GC-MS [Schmidt et al., 1988]. The selective ion monitoring (SIM) mode can be used to monitor extracts for the occurrence of endogenous compounds since this method can offer higher sensitivity than the scan mode.

LC-MS and LC-MS/MS techniques have provided new prospects for the investigation of polar and high–molecular-mass compounds without prior derivatization or hydrolysis. GA glucosyl esters can be detected by LC-electrospray ionization (ESI) MS in both positive and negative modes (Schneider and Schmidt, 1996). In the positive mode, the abundant $[M+Na]^+$ ion will be detected and enable sugar esters to be recognized from their molecular masses; another important fragment is the Na^+ adduct of the aglycone, $[M+Na\text{-sugar}]^+$, which will be useful for the aglycone identification [Schneider and Schmidt, 1996]. Under the negative mode, the molecular ion $[M-H]^-$ is weak but the $[M\text{-sugar}]^-$ ion, which represents the aglycone moiety, will be the base peak and enable us to identify aglycone easily.

Recently, a highly sensitive and high-throughput method for the simultaneous analysis of 43 molecular species of cytokinins, auxins, ABA and gibberellins with the new generation of LC-MS/MS was reported [Kojima et al., 2009]. They used an automatic liquid handling system for solid phase extraction, which enabled them to treat a large number of samples without handing mistakes. The ultra-performance liquid chromatography (UPLC) coupled with a tandem quadrupole mass spectrometer (qMS/MS) equipped with an electrospray interface (ESI; UPLC-ESI-qMS/MS) also provided the highest sensitivity comparing to other LC-MS systems. In order to improve the detection limit of negatively charged compounds, such as gibberellins, they derivatized the fractions containing auxin, ABA and gibberellins with bromocholine that has a quaternary ammonium functional group. Therefore, the negative charged compounds were converted to positive charged and analyzed with other originally positive charged compounds in a single run. This modification, called “MS-probe”, greatly increased the quantification limits of gibberellins and reduced the time for analysis. The quantification limits can reach 1 fmol for GA_1 and 5 fmol for GA_4.

1.1.3. Glycoside hydrolases

Enzymes that hydrolyze glycosidic bonds from the anomeric carbon of a sugar moiety are called glycoside hydrolases (GH). GH have been classified according to their enzymatic activities by the IUBMB Enzyme Commission, which assigned them E.C. numbers starting with 3.2.1, and by their sequence similarity or presumed evolutionary relationship, as was systemized by Henrissat [1991, Henrissat & Baroch 1993, 1996]. The latter approach allows analysis of large amounts of the sequence data that has become available with genomic sequencing projects and this can be used to hypothesize the functions of various gene products [International Rice Genome Project, 2005]. The down side of this relation-driven functional extrapolation is that it has led to much misannotation of the public databases due to assumptions of functions being made, and further studies of protein functions are necessary to determine their exact functions. Despite this, the grouping of related proteins, such as GH

into related groups that tend to have related functions facilitates the investigation of their functions. So far, one hundred and thirty-five GH families have been identified (CAZY, carbohydrate active enzyme website: www.cazy.org), and many of these have been grouped into clans of families that have similar 3-dimensional structures and, in general, utilize similar catalytic mechanisms [Coutinho & Henrissat, 1999; Cantarel et al., 2009]. Although they have similar sequences and mechanisms, members of GH families generally include enzymes with different substrate specificities. For instance, GH family 2 (GH2) contains β-galactosidases, β-glucuronidases and β-mannosidases, while GH3 contains exoglucanases, α-L-arabinosidases and β-xylosidases (CAZY).

1.1.3.1. Clan A β-glycosidases

GH Clan A β-glycosidases have catalytic domains that have $(\beta/\alpha)_8$-barrel conformations, typically with two catalytic carboxyl residues, the catalytic acid/base on the end of β-strand 4 and the nucleophile on the end of β-strand 7 of the barrel [Henrissat et al., 1995; Chuenchor et al., 2008]. Clan A enzymes generally catalyze hydrolysis by a shared retaining mechanism by which the stereochemistry of the anomeric carbon in the released sugar is conserved with that in the substrate. This is achieved by a two step mechanism in which the sugar is initially attacked by the catalytic nucleophile to displace the aglycone, which leaves with acid assistance from the catalytic acid/base, resulting in glycosylation of the enzyme. The covalent bond with the sugar residue is broken in the subsequent deglycosylation step in which a water molecule, or another nucleophile, attacks the anomeric carbon with basic assistance from the catalytic acid/base to displace the nucleophilic carboxyl residue. A large number of enzymes with diverse specificities fall in clan A, including those of GH families 1, 2, 5, 10, 17, 26, 30, 35, 39, 42, 50, 51, 53, 59, 72, 79, 86, 113, and 128, making it the largest clan in terms of numbers of families and different specificities. These enzymes are almost all specific to β-d-glycosidic linkages, some also hydrolyze α-L-arabinoside and α-L-iduronides, for which the glycone sugar residue is shaped similar to β-D-glycosidic residues. It is notable that these types of sugar linkages account for most of the structural polysaccharides of plants [Carpita, 1996; Cosgrove, 1997], as well as many glycosides in plant secondary metabolism [Chuankhayan et al., 2005].

1.1.3.2. Functions of plant β-glucosidases

In previous work, we annotated the GH1 and GH35 enzymes encoded in rice genomic sequences to begin to unravel the functions of these β-D-glycosidases in plants [Opassiri et al., 2006; Tantanuch et al., 2008]. β-Glucosidase functions that have been described include cell wall metabolism and recycling, phytohormone activation, release of defense compounds from inactive glycosides, activation of intermediates in metabolic pathways, and release of volatile compounds, such as fragrances [Ketudat Cairns and Esen, 2010]. They also play unknown roles in response to abiotic and biotic stresses and establishment of mutualistic relationships with endophytes. Some of these will be considered below.

1.1.3.2.1. Cell wall metabolism

Plant cell walls are assembled from cellulose, hemicelluloses, pectins and related polysaccharides, glycoproteins, and lignins, as well as boron, silica and other components, depending on the type of cell wall [Carpita, 1996; Cosgrove, 1997]. During growth and development, plants must constantly remodel and turnover their cell wall polysaccharides, and in processes like breakdown of endosperm cell walls in germination, they must completely disassemble them. This requires the action of endoglycosidases, especially

endoglucanases such as cellulases, but also the action of glycosidases to breakdown the released oligosaccharides to prevent them from inhibiting the endoglycosidases and to release the monosaccharide residues for use by the plant. In addition to release of sugar residues from oligosaccharides, some exoglycosidases can also act to release external residues from cell wall polysaccharides, making the polysaccharides more accessible to the endoglucanases, xylanases, galactanases and mannanases that hydrolyze the polysaccharide backbones. Therefore, plants generally have a wide range of enzymes with different specificities to act at different points in cell wall degradation and recycling.

Several rice GH1 enzymes hydrolyze β-linked oligosaccharides, suggesting this may be a major role for them in the plant [Akiyama et al., 1998; Opassiri et al., 2003, 2004, 2006; Kuntothom et al., 2009]. Rice BGlu1 (Os3BGlu7), Os3BGlu8 and Os7BGlu26 isoenzymes could also hydrolyze β-linked mannooligossacharides, as does a closely related HvβII enzyme from barley [Hrmova et al., 1996, 1998, 2006], suggesting they could have roles in recycling of multiple cell wall components. Another β-mannosidase/β-glucosidase has been described from Arabidopsis [Xu et al., 2004], suggesting this may be a common feature among plant exoglycosidases.

Many microorganisms metabolize plant cell walls as an energy source [Gilbert et al., 2008], and have developed a wide range of enzymes for cell wall breakdown and dedicated a significant portion of their genomes to this process. Such bacteria and fungi and enzymes derived from them are commonly used for biomass conversion. The opportunistic breakdown of plant cell walls by these organisms has evolved separately and is fundamentally different from that which occurs in plants.

Besides for their roles in cell wall breakdown, plant β-glucosidases also act in the development of secondary cell walls by their release of lignin precursors that can then participate in lignin formation [Dharmawardhana et al., 1995]. The presence of monolignol glucoside β-glucosidases has been shown in Arabidopsis [Escamilla-Treviño et al., 2006] and rice [Baiya et al., 2014], as well as pine trees [Dharmawardhana et al., 1995], and a cluster of 3 closely related genes and two gene fragments is also found in the rice genome [Opassiri et al., 2006]. Via this process, β-glucosidases may affect the mechanical properties and digestibility of cell wall by facilitating lignin production, in addition to their roles in polysaccharide recycling. Since decreasing lignin content is one of the main goals of genetic engineering of plants for bioconversion to fuels [Sticklen, 2006], the effect of these β-glucosidases on cell wall lignin content is of special interest.

1.1.3.2.2. Phytohormone activation

Many phytohormone glycosides and the β-glycosidases (mainly β -glucosidases) that hydrolyze them are found in plants, but their function in phytohormone regulation has been debated [Buchanan et al., 2014]. Phytohormone glycosides have been suggested to be deadend by-products of plant down-regulation of the phytohormones, but their high levels and availability suggest that they could be a source for rapid release of phytohormones in response to biotic or abiotic stress. Enzymes have been identified that can hydrolyze glycosides of gibberrilins [Schliemann, 1983], cytokinin [Brzobohatý et al., 1993], auxin [Jakubowska & Kowalczyk, 2005], jasmonic acid derivatives [Seto et al., 2009], and abscissic acid [Lee et al., 2006; Xu et al., 2012]. The demonstration that maize β-glucosidase could hydrolyze and activate cytokinin β-glucosides was taken as evidence for the role of β-glucosidases in phytohormone activation [Brzobohatý et al., 1993], but others noted that the more abundant DIMBOA-glucoside, which can serve as a defense compound, was likely the major substrate for this enzyme [Babcock & Esen, 1994]. However, the demonstration that an Arabidopsis β-

glucosidase could aggregate and be activated to release ABA in response to drought stress [Lee et al., 2006], suggests that at least some of these glycosylated forms are stored for the release by β-glucosidases under stress conditions. In rice, Schliemann [1984] demonstrated the presence of β-glucosidases that hydrolyzed gibberellin glucosides and glucosyl esters, but these enzymes have yet to be identified at the molecular level. Recently, it has also been shown that a rice enzyme can hydrolyze tuberillic acid (TA) glucoside (TAG), thereby modulating its level in the rice plant [Wakuta et al., 2010].

1.1.3.2.3. Defense

β-Glucosidases release toxic compounds from inactive glycosides in response to herbivory, fungal invasion and damage [Morant et al., 2008]. For instance, cyanogenic glycosides rapidly react to release HCN upon removal of their glucosyl blocking group by β-glucosidases [Poulton, 1990]. Another example is the release of hydroxaminic acids, like DIMBOA and DIBOA from their glucosides in maize [Babcock and Esen, 1994], wheat and rye [Sue et al., 2006] by β-glucosidases that mix upon tissue damage. In addition, the nonhost-type resistance of *Arabidopsis thaliana* to powdery mildew and other fungi has been shown to be mediated by PEN2, a peroxisomal thioglucosidase/β-glucosidase that hydrolyzes glucosinolates as part of the cellular response to fungal invasion [Lipka et al., 2005; Bednarek et al., 2009]. In addition, the endoplasmic reticulum body GH1 glycosyl hydrolase PYK10 is necessary for Arabidopsis to set-up a mutualistic interaction with the endophytic fungus *Piriformospora indica* [Sherameti et al., 2008]. In Arabidopsis lines deficient in PYK10, the *P. indica* overpopulates the roots and elicits different defensive proteins, suggesting that PYK10 may defend against overpopulation of the roots to achieve the balance needed for the mutualistic interaction.

Defense-related β-glucosidases and thioglucosidases are often stored in a different compartment from their glycoside substrates, and the two only come together upon compromising of the cell membranes during herbivory and microbial invasion [Morant et al., 2008]. For instance, the plant hydroxaminic acid glycoside β-glucosidases are stored in the chloroplasts, while their substrates are found in an alternative position, possibly the vacuole [Esen and Stetler, 1993; Nikus et al., 2003], as is also the case of saponin β-glucosidases of oat [Nisius, 1988]. The enzyme and substrate can also be in separate layers of cells or types of cells, as is seen in thioglucosidases or myrosinases, which are found in myrosin granuoles of myroblasts, while their substrates are found in other cells [Höglund et al., 1992]. Eudicotcyanogenic β-glucosidases are often found in the apoplast, while their substrates are found in the vacuole [Morant et al., 2008]. Defensive β-glucosidases are also often found in a different compartment from a cytoplasmic aggregating factor that binds to them upon cellular membrane disruption [Blanchard et al., 2001]. These proteins are thought to affect the localization after the cells are broken open to make the β-glucosidases more effective against herbivores and pathogens.

1.1.3.2.4. Response to abiotic and biotic stress

β-Glucosidases have been noted to be produced or activated in response to abiotic or biotic stress, although their roles are often unknown. For instance, ABA-GE β-glucosidase (designated AtBG1) is encoded by a cDNA isolated from a salt-stressed Arabidopsis subtraction library and was shown to hydrolyze ABA-GE [Lee et al., 2006], as mentioned above. Moreover, Arabidopsis plants with knockout mutations in this gene were impaired in the ability to close stomata in response to drought. AtBG1 was found to aggregate upon water deficiency, which resulted in a 4-fold increase in activity, suggesting a means by which active

ABA levels could be adjusted according environmental conditions. The mechanisms by which other β-glucosidases respond to stress are not so clear. Other β-glucosidases have been found to be upregulated in responses to stresses, including salt [Kawasaki et al., 2001], phosphate starvation [Malboobi & Lefebvre, 1997], and whitefly infestation [van de Ven et al., 2000].

1.1.4. Rice β-glucosidases

One of the first reports of partial characterization of rice β-glucosidases was the report of dwarf rice seedling β-glucosidases that could hydrolyze synthetic glucoconjugates by Schliemann [1984]. However, the β-glucosidases were apparently only partially purified and no molecular characterization was done. Akiyama and colleagues [1998] determined the N-terminal sequence of cell-wall-bound β-glucosidase that they purified from rice seedlings, which was active against cell-wall-derived oligosaccharides. Later, two β-glucosidase cDNA were cloned from rice seedlings and used to express recombinant proteins in *E. coli* [Opassiri et al., 2003]. The protein designated BGlu1 was found to be highly expressed in flower and shoot and also hydrolyzed β-(1,3)- and β-(1,4)-linked gluco-oligosaccharides. Further analysis showed that BGlu1 has 6 subsites for binding β-(1,4)-linked gluco-oligosaccharides and relatively high transglycosylation activity [Opassiri et al., 2004]. Subsequently, the rice genome sequences were analyzed and 40 glycoside hydrolase (GH) family 1 (GH1) genes were identified, although they included two genes likely to be derived from endophytes, two pseudogenes and two gene fragments [Opassiri et al., 2006]. Expressed sequence tag (EST) analysis showed that most of the remaining 34 genes are expressed in rice and that the gene for BGlu1 (*Os3bglu7*) was one of the most abundantly expressed. Other isoenzymes in the same phylogenetic cluster as Os3BGlu7, Os3BGlu8 and Os7BGlu26, were also found to hydrolyze oligosaccharides, but Os7BGlu26 preferred mannosides to glucosides, while Os3BGlu8 was more similar Os3BGlu7 [Kuntothom et al., 2009]. A cDNA for the gene that was most similar to the sequence of the cell-wall-bound β-glucosidase described by Akiyama et al. [1998], *Os4bglu12*, was cloned and used to express a recombinant protein, which was found to hydrolyze oligosaccharides, similar to Os3BGlu7. However, further it was found to hydrolyze *p*-nitrophenyl glycosides more efficiently and also hydrolyze steroid and flavonoid □-glucosides with similar efficiency [Opassiri et al., 2010], and it has even higher activity against salicylic acid glucoside [Hiromi et al., 2012]. Another isoenzyme, Os3BGlu6, has also been expressed and found to hydrolyze octyl β-D-glucoside and (1,3)- and (1,2)- β-linked disaccharides, but not cellooligosaccharides [Seshadri et al., 2009]. Currently, the structures of Os3BGlu6, Os3BGlu7, Os4BGlu12, and Os7BGlu26 have been determined and it appears that small differences in their active sites account for their differences substrate specificities.

The Arabidopsis β-glucosidases that have been shown to hydrolyze ABA-glucosyl ester (ABA-GE BG or BG1) and to be involved in plant microbe interactions (PYK10 and PEN2) belong to a Brassicaceae-specific phylogenetic cluster (Figure 1) [Opassiri et al., 2006], so the enzymes that might play similar functions in rice are yet to be discovered. Seto et al. [2009] also showed that rice makes β-glucoside of tuberonic acid (a jasmonic acid derivative), which can be hydrolyzed by a β-glucosidase. The isoenzyme most closely related to Os4BGlu12 (Os4BGlu13) was found to hydrolyze tuberonic acid beta-glucoside (TAG), and was designated TAG beta-glucosidase I (TAGG1) [Wikuta et al., 2010]. This, along with the gibberellin β-glucosidase work of Schlieman [1984] and the report of ABA-GE β-glucosidase in barley [Dietz et al, 2000] suggests that several rice β-glucosidases that hydrolyze phytohormones may exist.

1.2. Research Objectives (วัตถุประสงค์ของการวิจัย)

The objectives of this research project were:

1.2.1 Synthesis of gibberellin GA_4 glucosyl ester and glucoside for use in identifying gibberellin beta-glucosidases from rice extracts.

1.2.2 Identification of rice tissue extracts that contain high levels of beta-glucosidase that can hydrolyze the GA_4-glucoconjugates.

1.2.3 Purification of the rice GA_4 beta-glucosidases and identification of the isoenzymes hydrolyzing the gibberellin glucoconjugates.

1.2.4 Cloning and recombinant expression of rice beta-glucosidases and verification that they hydrolyze gibberellin glucoconjugates.

1.3. Scope of Research (ขอบเขตของการวิจัย)

The scope of this project was to synthesize the gibberellin beta-glucoconjugates and their use for beta-glucosidase identification. The β-glucosidases that hydrolyze these glucoconjugates enriched in purification fractions were identified by tryptic digest and mass spectrometery of sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE) bands and comparison of the peptide masses to predicted proteins from the rice genome database. The protein sequences were analyzed to see what properties, such as isoelectric point, may be used for further purification, while the cDNA were cloned or obtained from clones of full length cDNA clones from the Rice Genome Database or previously cloned in our lab. The sections of these cDNA encoding the predicted mature protein were cloned into protein expression vectors for expression of protein in *Escherichia coli* or *Pichia pastoris* and the activities of the recombinantly expressed proteins and/or the enzymes purified to homogeneity from rice were characterized for hydrolysis of gibberellin glucoconjugates and other glycosides available in our laboratory, as well as inhibition by various β-glucosidase inhibitors. In collaboration with Prof. Jong-Seong Jeon, Korea, we will also identify any T-DNA insertion lines for the identified β-glucosidase genes and these lines will be grown and inspected for the presence or absence of homozygotes and their growth characteristics.

1.4. Short Description of Methods (ข้อตกลงเบื้องต้น)

The production of gibberellin glucoconjugates were done by methods similar to those previously used for production of such compounds [Hiraga et al., 1974]. Reaction of gibberellins with equimolar amounts of alpha-acetobromoglucose in dichloroethane in the presence of Ag_2CO_3 followed by deacetylation gave the GA-β-D-glucosyl esters. By glucosylation of gibberellin methyl esters under similar conditions, but with an excess of the glucosyl donor, and by subsequent deacetylation and demethylation, the GA-glucopyranosides were obtained. The crude products were purified by flash column chromatography and HPLC. The structures of the synthesized compounds were confirmed by NMR and Mass spectrometry.

The synthesized GA-glucosyl conjugates were tested with the rice β-glucosidases which have been produced in our laboratory by TLC and glucose oxidase assays according to previous published methods [Opassiri et al., 2003, 2004]. Samples of substrate and protein were mixed well in 1.5 ml tubes and incubated at 30 °C for 20 min and overnight with a control without enzyme. The reaction were stopped by heating at 80 °C for 5 min, and then transferred to a microtiter plate for measurement of the glucose: 100 µl of peroxidase/glucose

oxidase enzyme and 50 µl of ABTS were added to each microtiter plate well and the plate were incubated at 37 °C for 30 min. The 405 nm absorbance were measured and compared to a glucose standard curve to quantify the amount of glucose [Opassiri et al., 2003].

Extraction, purification and characterization of rice enzymes which are able to hydrolyze the GA-glucosyl conjugates to active GAs followed previous published methods for screening of recombinant GH1 hydrolases already produced in our laboratory [Opassiri et al., 2003, 2006; Seshadri et al., 2009; Kuntothom et al, 2009], as were those of rice extracts [Schliemann, 1984]. For identification of the isoenzyme responsible for hydrolysis of these compounds in rice seedlings, the young rice seedling were homogenized in McIlvaine buffer and centrifuged for 20 min at 20,000 g. The pellets were washed by the same buffer three times and the combined supernatants were concentrated by ammonium sulfate precipitation (90% saturation). The precipitated protein were collected by centrifugation and redissolved in the McIlvaine buffer and loaded on a hydrophobic interaction column. The separation of the β-glucosidases was monitored with *para*-nitrophenol β-D-glucopyranoside (*p*NP-β-Glc) and GA-glucosyl conjugates as substrates. Fractions were eluted with a decreasing gradient of ammonium sulfate and those containing β-glucosidase activity were dialysed. The dialysed enzyme extract were clarified by short centrifugation and the soluble enzyme fraction were fractioned by CM-Sephadex C-50 or similar ion exchange column. The protein were further purified by a Superdex 200 gel-filtration column and eluted with NaCl and Tris–HCl. Those fractions containing the protein of interest were separated on SDS-PAGE and 2D electrophoresis (depending on the fraction complexity) and bands appearing to correspond to the activity were submitted to tryptic digest and mass spectrometry. The resulting peptide masses were compared to the proteins predicted from the rice genome to identify the isoenzyme involved. To assess the biochemical functions of putative gibberellin β-glucosidases, the cDNA for these proteins will be amplified from plasmids or reverse-transcribed RNA preparations and cloned into a Gateway entry vector and transferred to the pET32a/DEST and pPICZαBNH8/DEST expression vectors. If the cDNA encoding the proteins are available from the Rice Genome Resource Center (RGRC, Tsukuba, Japan), they will be used as templates to amplify the mature-protein encoding section of the cDNA for expressing the protein. Otherwise, the gene expression will be evaluated by inspection of the sources of RNA for ESTs and cDNA in the public databases. The RNA will be extracted from the appropriate tissue with a plant RNA extraction kit and reverse treanscribed from a poly-T or specific reverse primer to give the first strand cDNA. Primers will be designed for amplification of the full-length protein encoding cDNA from the start codon and the mature protein encoding region, as predicted by SignalP [Petersen et al., 2011], to the stop codon. The full-length protein-encoding region cDNA will be amplified by PCR from reverse-transcription-derived cDNA with *Pfu* polymerase. The cDNA will be gel purified and cloned into pENTR-D/Topo by topoisomerase reaction, according to the supplier's instructions (Invitrogen). The entry clones will be sequenced to verify the correct sequence and transferred into the pET32/DEST and pPICZαBNH8/DEST plasmids by LR Clonase recombination (Invitrogen). The *E. coli* expression vectors will be transformed into Origami(DE3), Origami B(DE3) and/or Rosetta-gami(DE3) *E. coli* and screened for expression of protein with induction with varying amounts of isopropy β-D-thiogalactoside (IPTG) at 20 °C, as previously described [Opassiri et al., 2003; 2006; Seshadri et al., 2009; Kuntothom et al., 2009]. Note that all these strains provide for disulfide formation in the cytoplasm and synthesis of mRNA by T7 polymerase and Rosetta-gami cells have extra tRNA genes for codons uncommonly found in *E. coli*. If active soluble protein is produced, it will be purified by immobilized metal affinity chromatography (IMAC), digestion with

enterokinase, TEV or thrombin protease, and a second IMAC column, and/or ion exchange, hydrophobic interaction and gel filtration chromatography, as necessary. If the enzymes cannot be produced in *E. coli*, they will be expressed in *Pichia pastoris* from the pPICZαBNH8 plasmids, and purified. The pPICZαBNH8 constructs verified by sequencing, linearized and transformed into *P. pastoris* by electroporation. The protein will be expressed in *P. pastoris* and the proteins purified by ion exchange chromatography and IMAC as described by Luang et al [2010]. If the protein cannot be produced from the cDNA in either system, a synthetic gene that is optimized for expression in *P. pastoris* will be ordered and cloned into pPICZαBNH8, as described for barley ExoI exoglucanase [Luang et al., 2010]. Protease deficient cells and low temperature (20 °C) will be tested to see if protein production can be improved under these conditions.In test whether the GA-glucoconjugates are rapidly metabolized and utilized in the plant and whether the enzymes that hydrolyze them play significant roles in GA action, the GA-glucosyl conjugates and GA-inhibitors will be fed to rice seedlings. The seedlings will be grown under the light or in the dark for 7 days, and their height/length will be measured and analyzed for a correlation of growth to the presence of substrates. In order to analyze the metabolism of GA gluco-conjugates in the plant, gibberellin metabolites will be extracted from rice seedlings after feeding GA-glucosyl conjugates and quantified by HPLC, LC-MS and GC-MS, following published methods [Schneider G, et al 1992, Cassán, et al., 2001]. The rice seedlings will be grounded in liquid nitrogen to powder and extracted with MeOH. The extracts will be dried and loaded onto DEAE-Sephadex A-25 column and eluted with the mixtures of MeOH with acetic acid. The acidic fractions will be combined, evaporated to dryness, and subsequently purified by HPLC. The free GA fractions from HPLC will be methylated and silylated, then analyzed by GC-MS. The metabolic pathway will be interpreted according to the metabolites identified from GC-MS or LC-MS [Kojima et al., 2009]. This may need to be done in collaboration with researchers with high level LC-MS-MS equipment. To identify the effects of knockout of the genes, we will collaborate with Jong-Seong Jeon of Kyung He University, Korea, to identify T-DNA knock-down lines of the genes encoding the proteins. If such lines are identified in a database search, the rice will be grown and the presence or absence of the insertion in the progeny plants will be assessed by PCR with primers bracketing the insertion site and with one primer from the gene and a second primer from the T-DNA insertion to see whether wildtype and T-DNA inserted genes are present in the plant. The number of homozygous and heterozygous plants will be assessed to check the effect of gene knockdown on plant viability. Then the phenotypes of wildtype, heterozygous and homozygous plants will be compared in terms of growth rates, final height and seed germination rates.

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

1.5.1. Knowledge. This work discovered that the enzymes which could hydrolyze GA_4-glucosyl ester to GA_4 exist in the rice glumes, seeds, seedlings and roots, though the pure enzymes were not separated in this project yet. Os3BGlu6 was found to have the highest hydrolysis activity to GA_4-Glc among five rice GH1 enzymes that have been expressed in our lab, compared to Os3BGlu7 (BGlu1), Os4BGlu12, Os3BGlu18 and Os9BGlu31. So, Os3BGlu6 could be a good model for investigation of β-glucosidase hydrolysis of glucosyl esters, since little description of glucosyl ester hydrolysis is available in the literature.

1.5.2. Human Resources Development. One Ph.D. student and one research assistant have been trained on this project and learned more skills.

1.5.3. Research Publication. This work was presented as poster at **The 6th International Symposium of Protein Society of Thailand**. Part of the work from this project was combined with other research work and published 2 papers in the journal **Arch. Bichem. Biophys.** [1. Hua Y, Sansenya S, Saetang C, Wakuta S and Ketudat Cairns JR (2013). Enzymatic and structural characterization of hydrolysis of gibberellin A_4 glucosyl ester by a rice β-D-glucosidase. Archives of Biochemistry and Biophysics**.** 537(1): 39-48; 2. Hua Y, Ekkhara W, Sansenya S, Srisomsap C, Roytrakul S, Saburi W, Takeda R, Matsuura H, Mori H, Ketudat Cairns JR (2015). Identification of rice Os4BGlu13 as a β-glucosidase which hydrolyzes gibberellin A4 1-O-β-D-glucosyl ester, in addition to tuberonic acid glucoside and salicylic acid derivative glucosides. Archives of Biochemistry and Biophysics. 583: 36-46.]

CHAPTER 2. Materials and Methods

2.1 Materials

2.1.1 Plant material

Rice (*Oryza sativa* cv. Suphan buri 1) was grown in a field in Sikiu district, Nakhon Ratchasima province during December, 2011. After 10 days, seedling shoots and leaves were collected and used for protein purification.

2.1.2 Chemicals and laboratory supplies

Gibberellic acid GA_3 and gibberellin GA_4 were purchased from Jiangsu Fengyuan Bioengineering Co. Ltd. (Sheyang, P. R. China). *p*-toluenesulphonylmethylnitrosamide (Diazald) was purchased from Shanghai Jinglan Chemical Co. Ltd (Shanghai, P. R. China). Quinoline, α-aceto-bromoglucose, *p*-nitrophenyl-β-D-glucopyranoside (*p*NPGlc), peroxidase/glucose oxidase assay (PGO), phenylmethylsulfonyl fluoride (PMSF), sea sand, 2,2'-azino-bis(3-ethyl-benzothiazoline-6-sulphonic acid) (ABTS), ampicillin, DNase I, kanamycin, tetracyclin, isopropyl β-D-thiogalactoside (IPTG) and lysozyme were purchased from Sigma (St. Louis, USA). Silver oxide, methanol-d4, acetone-d6, chloroform-d, pyridine-d5, deuterium oxide and tetramethylsilane (TMS) were purchased from Aldrich (St. Louis, USA). Molecular sieve 4 Å, trifluoroacetic acid (TFA), polyethylene glycol (PEG), 2-morpholinoethanesulfonic acid (MES), Triton X-100, bovine serum albumin (BSA), calcium chloride, metal sodium and formic acid were purchased from Fluka (Steinheim, Switzerland). 1,4-Dioxane was purchased from Fisher Scientific (Aalst, Belgium). Silica gel 60 and silica gel 60 F_{254} TLC plates were purchased from Merck (Darmstad, Germany). HPLC-grade water and HPLC-grade methanol were purchased from RCI Labscan (Bangkok, Thailand). Trypsin (sequencing grade) was purchased from Promega (Madison, WI, USA). The Bradford assay kit was purchased from Bio-Rad (Hercules, CA, USA). Imidazole was purchased from USB Corporation (Cleveland, OH, USA). Dichloroethane, benzene, ethyl acetate, dichloromethane, chloroform, acetic acid, sulfuric acid, hexane, methanol, ethanol, acetone, pyridine, isopropanol, *n*-propanol, acetonitrile, tetrahydrofuran, disodium ethylenediamine tetraacetate (EDTA), bromophenol blue, ammonium bicarbonate, calcium chloride (anhydrous), sodium sulfate (anhydrous), magnesium sulfate (anhydrous), ammonium sulfate, sodium bicarbonate, sodium chloride, sodium hydroxide, sodium carbonate, citric acid, disodium hydrogen phosphate, hydrochloric acid, Coomassie brilliant blue R250, Tris(hydroxymethyl)-aminomethane (Tris) and sodium dodecyl sulfate were purchased from CARLO ERBA (Rodano, Milano, Italy). Acrylamide, N,N′,N″,N‴-tetramethyl-ethyllenediamine (TEMED), ammonium persulphate, N,N′-methylenebisacrylamide, ConA-Sepharose resin, Superdex-75 and Superdex-200 gel filtration resin, immobilized metal affinity chromatography (IMAC) resin, HiPrep CM-Sepharose fast flow column (16/10, 20 ml), HiTrap SP Sepharose XL column (1 ml) and HiTrap Octyl Sepharose 4 fast flow column (1 ml) were purchased from GE Healthcare (Uppsala, Sweden). Dialysis bags (Cellu-SepT4, regenerated cellulose, 12,000-14,000 MWCO) were purchased from Membrane Filtration Products, Inc. (Seguin, TX, USA). QuikChange® Site-Directed Mutagenesis Kit was purchased from Stratagene (La Jolla, CA, USA). Ultra centrifugal filters (Amicon Ultra, regenerated cellulose, 30,000 MWCO) were purchased from Millipore Corporation (Bedford, MA, USA). Other chemicals and laboratory materials used but not listed here were purchased from a variety of suppliers.

2.2 Experimental methods

2.2.1 Synthesis of gibberellin glucosyl conjugates

2.2.1.1 Synthesis of glucosyl esters of gibberellin GA_3 and GA_4

The GA_3-glucosyl ester (GA_3-GE) and GA_4-glucosyl ester (GA_4-GE) were synthesized from 3 mmol of gibberellin GA_3 or GA_4 α-acetobromo-glucose tetraacetate following the method of Hiraga et al. (1974, Figure 2.1). Acetylated GA_4-GE was produced with 43.7% yield (0.870 g), while acetylated GA_3-GE was obtained in 18.3% yield.

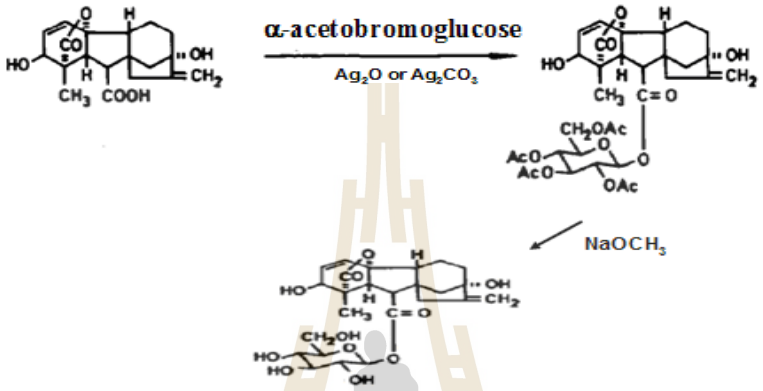

Figure 2.1 Reaction scheme for synthesis of gibberellin glucosyl esters (Hiraga et al., 1974).

Acetylated GA_4-GE (0.733 g) was deacetylated by dissolving it in 15 ml of methanol (MeOH), cooling to -5°C, and adding 75 ml of 0.05 N sodium methoxide (NaOMe). After 2 hours reaction, the solution pH was adjusted to pH 7 with acetic acid, and the solvent was removed by concentration in a workstation -TurboVap® LV (Caliper life Science, USA) under N_2 flushing at room temperature. The solid obtained was purified by flash silica column chromatography with chloroform/methanol ($CHCl_3$/MeOH) to yield 0.504 g of GA_4-GE (92.3% yield), while GA_3-GE was obtained in 60% yield.

2.2.1.2 Identification of synthesized products with LC-MS and NMR

An Agilent 1100 HPLC equipped with a ZORBAX Eclipse XDB-C18, 4.6*150 mm, 5 micron column (Agilent, USA) was used to separate the samples. A gradient of 0-80% MeOH in 0.05% (v/v) formic acid was run over 20 min at a flow rate of 0.8 ml/min. The ion peaks and mass spectra were detected with an Agilent single quadrupole MSD mass spectrometer with the atmospheric pressure ionization-electro spray (API-ES) source in negative and positive ion modes. The scan range was 100-1000 *m/z*, and the fragmentor voltage was 70 V. The flow rate of drying gas was 12.0 l/min and the temperature of the gas was 300°C. The VCap was 3000 V for both positive and negative modes.

The synthesized compounds were confirmed by NMR spectra on a 300 MHz NMR spectrometer (Unity INOVA, Varian, USA). Tetramethylsilane (TMS) was used as the reference standard. Deuterated chloroform ($CDCl_3$), acetone-d6 and methyl sulfoxide-d_6 (DMSO-d_6) were used as solvents, depending on the compound's solubility. The NMR spectra were collected with a Varian 300 ID/PFG probe at a frequency of 299.986 MHz. The software VNMR version 6.1 was used for data processing.

2.2.2 Extraction, purification and characterization of β-glucosidase from rice

2.2.2.1 Extraction of β-glucosidase from 10-day-old rice seedlings

Ten kilograms of 10-day-old rice seedling shoots and leaves (Suphan buri 1) were cut to small pieces with a blender, homogenized with McIlvaine buffer (0.1 M citric acid-0.2 M disodium hydrogen phosphate (Na_2HPO_4), pH 5.0) supplemented with 1 mM phenylmethylsulfonyl fluoride (PMSF) at 4°C overnight. The ratio of seedlings and buffer was 100 g per 600 ml. A crude extract was obtained by filtering through one layer of silk cloth and centrifugation at 12,000 g for 20 min at 4°C. To precipitate protein, 565 g of ammonium sulphate $(NH_4)_2SO_4$ was added per one liter of crude extract (80% saturation) and stirred at 4°C for 3 h. The solution was centrifuged at 12,000 g for 20 min at 4°C to collect the protein pellet. The protein pellet was suspended in 4-fold diluted McIlvaine buffer, pH 5, and dialyzed overnight against this buffer in dialysis bags (Cellu-SepT4, regenerated cellulose, 12,000-14,000 MWCO, USA). The dialysis solution was exchanged once. The dialyzed protein was centrifuged at 12,000 g for 20 min at 4°C again to remove precipitate. The supernatant was tested for hydrolysis activities toward *p*NPGlc and GA_4-Glc, and the fractions with the GA_4-Glc hydrolyzing activity were purified with the following procedures.

2.2.2.2 Purification of β-glucosidase by ion exchange chromatography with a CM-Sepharose fast flow column

A CM-Sepharose fast flow column (HiPrep CM FF 16/10, 20 ml, GE Healthcare) was equilibrated with 4-fold diluted McIlvaine buffer, pH 5 (buffer A). About 400 mg of dialyzed protein was loaded on to the column. The unbound proteins were washed out with buffer A, and then the bound proteins were eluted with a linear gradient of 0-1.0 M sodium chloride (NaCl) in buffer A at a flow rate of 2.0 ml/min. The column was cleaned with 1.0 M NaCl in buffer A, and then re-equilibrated with buffer A for the next purification. The fractions were collected and tested for hydrolysis activities toward *p*NPGlc and GA_4-GE. The fractions with activities to GA_4-GE were pooled and precipitated with $(NH_4)_2SO_4$ (80% saturation), followed by centrifugation at 16,000 g for 20 min at 4°C, then dialyzed against 20 mM Tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl), pH 7, containing 0.5 M NaCl (buffer B).

2.2.2.3 Purification of β-glucosidase by affinity chromatography with a Con A-Sepharose column

Ten milliliters of Con A-Sepharose 4B was packed in an empty glass column (GE Healthcare) and equilibrated with buffer B. Then, 200 mg of dialyzed protein was loaded onto the column. The column was washed with 4 column volumes (CV) of buffer B, and then eluted with 4 CV of 0.5 M mannose in buffer B. Four CV of buffer B were used to wash the column again. Then, 20 mM Tris-HCl, containing 0.5 M of NaCl, pH 8.5 and pH 4.5 were used to clean and re-generate the Con A-Sepharose column. The collected fractions were tested for hydrolysis of *p*NPGlc as mentioned above; the active fractions were combined and concentrated with centrifugal filters (Amicon Ultra, regenerated cellulose, 30,000 MWCO) at 2,800 rpm, 4°C. The buffer of the concentrate was exchanged twice with 4-fold diluted McIlvaine buffer, pH 7, containing 0.2 M NaCl (buffer C), before testing activity with GA_4-GE and further purification by gel filtration chromatography.

2.2.2.4 Purification of β-glucosidase by gel filtration chromatography with a Superdex-75 gel filtration column

A Superdex-75 gel filtration column (xk 26/40, 150 ml, GE Healthcare) was equilibrated with 2 CV of buffer C on an ÄKTA Protein Purifier system (GE Healthcare). Three milliliters of concentrated protein from the Con A-Sepharose column was loaded to the column with a super loop. Buffer C was used to elute the column at a flow rate of 1.0 ml/min. The fractions were tested for *p*NPGlc and GA_4-GE hydrolysis activities and their purities checked with polyacrylamide gel electrophoresis (SDS-PAGE). The active fractions were pooled and concentrated with centrifugal filters as described above, then the buffer was exchanged with 4-fold diluted McIlvaine buffer, pH 5 (buffer A).

2.2.2.5 Purification of β-glucosidase by cation exchange chromatography with a HiTrap SP XL column

A 1 ml HiTrap SP Sepharose XL column (GE Healthcare) was equilibrated with buffer A on an ÄKTA Protein Purifier system. Five hundred microliters of concentrated protein from the S-75 gel filtration column β-glucosidase pool was loaded onto the column. The column was eluted with a linear gradient of 0-1.0 M NaCl in buffer A at a flow rate of 1.0 ml/min. The fractions were tested for *p*NPGlc and GA_4-GE hydrolysis activities. The purities of the active fractions were checked with SDS-PAGE and then similar fractions were pooled, concentrated with centrifugal filters as described above, and the buffer exchanged with 50 mM phosphate, pH 7, containing 1.7 M $(NH_4)_2SO_4$ (buffer D).

2.2.2.6 Purification of β-glucosidase by hydrophobic interaction chromatography with an Octyl Sepharose 4 column

Three milliliters of dialyzed protein from SP chromatography were loaded to a 1 ml HiTrap octyl FF column (Sepharose 4, GE Healthcare), which was pre-equilibrated with buffer D. The protein was eluted with a linear gradient of 1.7-0 M of $(NH_4)_2SO_4$ in 50 mM phosphate, pH 7. The buffer of the collected fractions was exchanged twice with 4-fold diluted McIlvaine buffer, pH 5. The fractions were then tested for *p*NPGlc and GA_4-GE hydrolysis, and their protein components evaluated with SDS-PAGE.

2.2.2.7 Purification of β-glucosidase by gel filtration chromatography with a Superdex-200 gel filtration column

The active fractions from the octyl Sepharose 4 column were concentrated and loaded to the Superdex-200 gel filtration column (10/300, 24 ml, GE Healthcare) which was equilibrated with buffer C, as described for the S-75 gel filtration column, and eluted with the same buffer. The protein concentrations and GA_4-GE hydrolysis activities of the fractions were measured, and their protein compositions checked with SDS-PAGE.

2.2.2.8 Identification of β-glucosidases with LC-MS of tryptic peptides

The protein from the Superdex-200 column purification was separated on an 8% SDS-PAGE. Two main bands were exercised separately and chopped to 5-8 pieces (1x1x1 mm), then destained with 25 mM ammonium bicarbonate (NH_4HCO_3)/50% methanol (v/v). Two hundred microliters of sterile water was added into the vials and they were shaken for 5 min at room temperature. Water was removed and 200 µl of 100% acetonitrile (ACN) was added and shaken for 5 min at room temperature. After the ACN was removed, the gel plugs were dried at room temperature for 5-10 min. In order to reduce disulfide bonds, the gels were immersed in 20 µl of 10 mM dithiothreitol in 10 mM NH_4HCO_3 and incubated at 56°C for 1 h. After removal of the 10 mM dithiothreitol in 10 mM NH_4HCO_3 solution, 20 µl of 100 mM

iodoacetamide in 10 mM NH_4HCO_3 was added to the gels. The gels were kept in the dark at room temperature for 1 h, and then washed twice with 200 µl of 100% ACN.

Twenty microliters of 10 ng/µl trypsin (Promega, sequencing grade) was added to the gels for digestion. The gels were kept at 4ºC or room temperature for 20 min then incubated at 37°C for 3 h. The solution was transferred to a new tube, extracted with 30 µL of 50% ACN/0.1% formic acid (v/v), and shaken at room temperature for 10 min. The extracted solution was dried in an incubator at 40°C for 3-4 h or overnight. The samples were kept at -80°C until analysis.

Nanoscale LC separation of tryptic peptides was performed with a NanoAcquity system (Waters Corp., Milford, MA) equipped with a Symmetry C_{18} 5 µm, 180 µm x 20 mm trap column and a BEH130 C_{18} 1.7 µm, 100 µm x 100 mm analytical reverse phase column (Waters Corp., Milford, MA)at 35°C. The samples were initially transferred with an aqueous 0.1% formic acid solution to the trap column with a flow rate of 15 µl/min for 1 min. Mobile phase A was 0.1% formic acid in water, while mobile phase B was 0.1% formic acid in ACN. The peptides were separated with a gradient of 15–50% mobile phase B over 15 min at a flow rate of 600 nl/min followed by a 3-min rinse with 80% of mobile phase B. The lock mass was delivered from the auxiliary pump of the NanoAcquity pump with a constant flow rate of 500 nl/min at a concentration of 200 fmol/µl of [Glu^1]fibrinopeptide B to the reference sprayer of the NanoLockSpray source of the mass spectrometer. All samples were analyzed once. Analysis of tryptic peptides was performed on a SYNAPT^{TM} HDMS mass spectrometer (Waters Corp., Manchester, UK). For all measurements, the mass spectrometer was operated in the V-mode of analysis with a resolution of at 9,000 full-width half-maximum. All analyses were performed in positive ion nanoelectrospray mode. The time-of-flight analyzer of the mass spectrometer was externally calibrated with [Glu^1]fibrinopeptide B from *m/z* 50 to 1600 with acquisition lock mass corrected using the monoisotopic mass of the doubly charged precursor of [Glu^1]fibrinopeptide B. The reference sprayer was sampled with a frequency of 20 s. Accurate mass LC-MS data were acquired in data direct acquisition mode. The energy of the trap was set at a collision energy of 6 V. In the transfer collision energy control, the low energy was set at 4 V. The quadrupole mass analyzer was adjusted such that ions from *m/z* 300 to 1800 were efficiently transmitted. The MS/MS survey was over the range of 50 to 1990 Da and the scan time was 0.5 s.

2.2.2.9 Determination of β-glucosidase activity

The activities of protein fractions to hydrolyze *p*NPGlc were tested in a manner similar to previously published methods (Opassiri et al., 2003, 2006; Seshadri et al., 2009; Kuntothom et al., 2009). Aliquots of enzyme solutions were incubated with 4 mM *p*NPGlc in 50 mM sodium acetate (NaOAc) buffer, pH 5.0, (total reaction volume 50 µl) at 30°C for 20 min. The reactions were stopped by adding 150 µl of 2 M sodium carbonate (Na_2CO_3). The released *p*-nitrophenol (*p*NP) was quantified by measuring the absorbance at 405 nm (A_{405}) with a microplate reader (Thermo Labsystems, Finland), and comparing it to that of a *p*NP standard curve.

The hydrolysis of GA_4-glucosyl ester (GA_4-GE) was determined with a peroxidase/glucose oxidase-based assay (PGO assay, Sigma). PGO reagent was prepared by dissolving one capsule of PGO enzymes in 100 ml of sterile distilled water. 2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) was dissolved to 1 mg/ml in 50 mM NaOAc buffer, pH 5.0. The protein fractions were incubated with 1.72 mM GA_4-GE in 50 mM NaOAc buffer, pH 5.0, at 30°C for 20 min. The reactions were stopped by boiling 1 min and

cooled on ice immediately. Then, 100 µl of PGO and 50 µl of ABTS were added to the reactions, mixed and incubated at 37°C for 30 min. The A_{405} was measured, and the amounts of glucose released were calculated from a glucose standard curve developed in the same manner. Protein concentrations were determined with a Bio-Rad Bradford assay with bovine serum albumin (BSA) as a standard.

2.2.2.10 Determination of protein components, purity and size with SDS-PAGE

SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis was performed according to Laemmli [1970]. 12.5%, 10% and 8% (w/v) acrylamide separating gels were prepared in 0.375 M Tris-HCl buffer, pH 8.8, while 5% stacking gel was prepared in 0.065 M Tris-HCl buffer, pH 6.8. Fifteen microliters of protein was mixed with 10 µl of reducing loading buffer (0.05 M Tris-HCl buffer, pH 6.8; 50% glycerol (v/v); 10% SDS (w/v); 20% 2-mercaptoethanol (v/v) and 0.2 mg/ml bromophenol blue), boiled 5 min before loading onto the gel. The protein standard marker, which included bovine α-lactalbumin (14 kDa), trypsin inhibitor (21.5 kDa), bovine carbonic anhydrase (31 kDa), ovalbumin (45 kDa), BSA (66 kDa) and phosphoryase (97.4 kDa), was loaded on to the same gel. The gel was stained with Coomassie brilliant blue R-250 (CBB) solution for 40 min, which included 0.025% CBB, 40% methanol, 7% acetic acid, and then destained with destaining solution (40% methanol and 7% acetic acid) twice, followed by water. Silver staining was also used to detect low amounts of protein. The silver staining kit from GE Healthcare was used following the company's protocol.

2.2.3 Screening of rice GH1 enzymes for GA_4-glucosyl ester hydrolysis

Five glycoside hydrolase family 1 (GH1) enzymes that have been expressed in our lab, Os3BGlu6 [Seshadri et al., 2009], Os3BGlu7 [BGlu1, Opassiri et al., 2003], Os4BGlu12 [Opassiri et al., 2006], Os4BGlu18 [Baiya et al.,2014] and Os9BGlu31 [Luang et al.,2013] were tested for the hydrolysis activity to *p*NPGlc and GA_4-GE according to method described in section 2.2.2.9.

2.2.4 Site-directed mutagenesis of Os3BGlu6

Mutagenesis of the pET32/Os3BGlu6 expression vector [Seshadri et al., 2009] to create the Os3BGlu6E178A, Os3BGlu6E178D, Os3BGlu6E178Q, Os3BGlu6E394D and Os3BGlu6E394Q mutations was performed with the QuikChange® Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA) according to the supplier's instructions. The following oligonucleotides were used for mutagenesis: for E178A, 5'GATCACGCTCAA CGCGCCGCACACGGTG3' and its reverse complement; for E178D, 5'GATCACGCT CAACGATCCGCACACGGTGG3' and its reverse complement; for E178Q, 5'GGATCA CGCTCAACCAACCGCACACGGTGGC3' and its reverse complement; for E394D, 5'CCA GTGTACATCACTGATAACGGGATGGATGACA GC3' and its reverse complement; and for E394Q, 5'CCACCAGTGTACATCACTCAGAACGGGATGGATGA CAGC3' and its reverse complement. The cDNA were confirmed to include the desired mutations and be free of additional mutations by automated DNA sequencing (Macrogen Corp., Seoul, Rep. of Korea).

2.2.5 Recombinant expression and purification of Os3BGlu6 and its mutants

2.2.5.1 Recombinant expression of Os3BGlu6 and its mutants

The wild type rice Os3BGlu6 and its mutants M251N, E178Q, E178A and E178D were expressed in *Escherichia coli* strain Origami(DE3) as fusion proteins with N-terminal

thioredoxin and His_6 tags at 20°C for 16-18 h, as described previously for Os3BGlu6 [Seshadri, et al., 2009]. Cells were collected by centrifugation at 5,000 rpm for 10 min at 4°C and the pellet was stored at -80°C for 30 min or until protein extraction.

2.2.5.2 Extraction of recombinant Os3BGlu6 protein from induced cell

The pellets were thawed at room temperature. Cells were resuspended with extraction buffer (20 mM Tris-HCl, pH 8.0, 200 µg/ml lysozyme, 1% Triton-X100, 1 mM PMSF and 0.25 mg/ml DNase I) and incubated at room temperature for 30 min. Soluble proteins were separated from cell debris by centrifugation at 12,000 rpm for 10 min at 4°C.

2.2.5.3 Purification of Os3BGlu6 and its mutants

Os3BGlu6 wild type, E178A, E178D, E178Q and M251N were purified with 2 steps of IMAC. The crude protein was mixed with pre-equilibrated cobalt (IMAC) resin with equilibration buffer (20 mM Tris-HCl, pH 8.0, and 150 mM NaCl) at 4°C for 30 min. The resin with crude protein was loaded into a column and unbound proteins were washed out with 5 CV of equilibration buffer, and 5 CV each of 5 mM and 10 mM imidazole in equilibration buffer. The bound proteins were eluted with 5 CV of 250 mM imidazole in equilibration buffer. The fractions with activity were combined, concentrated and imidazole removed by centrifugal ultrafiltration (Amico Ultra, 30k MWCO) at 2,800 rpm, 4°C; and the buffer exchanged with 50 mM Tris-HCl, pH 8.0, at 4°C. The concentrated proteins from the 1st IMAC step were incubated with Tobacco etch virus (TEV) protease at 4°C for 12-14 h to cut off the N-terminal thioredoxin, His_6 and S tags. The N-terminal thioredoxin, His_6 and S tags were removed with a second IMAC purification.

2.2.6 Characterization of Os3BGlu6 and its mutants

2.2.6.1 Determination of pH optima for Os3BGlu6 and its mutants

The pH optima of Os3BGlu6 and Os3BGlu6 M251N for hydrolysis of *p*NPGlc were determined by incubating 1 µg of enzyme with 2 mM *p*NPGlc, in 80 µl of universal buffer (0.1 M citric acid-0.1 M disodium hydrogen phosphate, final volume 100 µl), pH 2 to 11 in 0.5-pH-unit increments, at 30°C for 10 min. The reactions were stopped by adding 100 µl of 2 M sodium carbonate (Na_2CO_3). The released *p*NP was determined quantitatively by measuring the absorbance at 405 nm (A_{405}) and calculated by comparison to a *p*NP standard curve.

The optimum pH of Os3BGlu6 and its M251N, E178Q and E178A mutants for hydrolysis of GA_4-GE were determined by incubating 1 µg of Os3BGlu6 or Os3BGlu6 M251N, or 5.0 µg of E178Q or E178A with 0.86 mM GA_4-GE in 80 µl of 100 mM universal buffer (final volume was 100 µl), pH 2 to 11 in 0.5-pH-unit increments at 30° for 20 min. The reactions were stopped by boiling 1 min and cooled on ice immediately. Then, the amounts of glucose released were determined by the PGO assay as described in section 2.2.2.9.

The activity versus pH curves of the E178 Q and E178A mutants for hydrolysis GA_4-GE were also determined in 50 mM MES buffer in the range of pH 5.0 to 7.5 in 0.5-pH-unit increments, following the same procedure as in universal buffer.

2.2.6.2 Measurement of wild type and mutant Os3BGlu6 activities for hydrolysis of gibberellin glucosyl ester and *p*NP-glucoside

The activities of Os3BGlu6 wild type and mutants to hydrolyze *p*NPGlc and GA_4-GE were determined as described in section 2.2.2.9.To determine the kinetic parameters, variable reaction times, enzyme amounts and substrate concentrations were tested to obtain the initial

velocities. The K_m and k_{cat} were calculated from nonlinear regression of Michaelis-Menten plots with Grafit 5.0 software.

2.2.6.3 Identification of transglucosylation products with TLC, LC-MS and NMR

Transglucosylation reactions were studied for Os3BGlu6 wild type and its M251N, E178Q and E178A mutants. For Os3BGlu6 wild type and E178Q mutant, *p*NPGlc or 2',4'-dinitrophenyl-2-deoxy-2-fluoro-β-D-glucopyranoside (2F-DNPG) donor was reacted with 1.0 µg of enzymes in 50 mM MES buffer, pH 5; and 40 mM sodium azide or 0.96 mM free GA_4 was used as acceptor. At different times, 10 µl aliquots of reaction mix were removed, boiled 1 min, and kept on ice for TLC analysis. Reactions without enzymes were used as controls. After overnight reactions, sample aliquots were spotted on TLC and the TLC plates developed with EtOAc-MeOH-H_2O (7.5:2.5:1.0, v:v:v) or $CHCl_3$-MeOH (7:3, v:v). The products were detected by staining with 10% (v/v) sulfuric acid in ethanol followed by charring.

The new transglucosylation products of E178Q and E178A were analyzed with LC-MS. The column and LC-MS method was the same as that described in section 2.2.1.4.

The transglucosylation product was also manually collected from HPLC with multiple injections. The fractions were pooled and dried with a vacuum centrifuge. The product was dissolved in acetone-d6, and ^{1}H NMR and gCOSY spectra were collected with a 300 MHz NMR spectrometer (Unity INOVA, Varian, USA). TMS was used as the reference standard.

2.2.6.4 Transglucosylation kinetics of the acid/base mutants of Os3BGlu6

The pH optima of Os3BGlu6 E178Q and E178A for transglucosylation were determined in 50 mM MES, pH 5.0-7.5, and 50 mM NaOAc buffer, pH 4.0-6.0, in 0.5-pH-unit increments. Two micrograms of E178Q or E178A were incubated with 2 mM GA_4-Glc and 100 mM sodium azide, in the different pH buffers at 30°C for 30 min. The reactions without sodium azide were used to measure hydrolysis activities. The reactions were stopped by boiling 1 min and cooled on ice immediately. The reaction mixes were centrifuged at 6,000 rpm for 15 min and separated by LC-MS, as described in the preceding section. The gibberellin GA_4 released was detected with a diode array detector (DAD) at 210 nm, and the amounts were calculated by comparison of the peak areas to a gibberellin GA_4 standard curve.

The concentrations of donor GA_4-GE and acceptor sodium azide were varied to test their effects on transglucosylation kinetics. The concentration of sodium azide was varied from 0 to 400 mM, while the GA_4-GE donor was fixed at 2 mM. The reactions were performed in 50 mM MES, pH 5, at 30°C for 20 min. The turnover rates of GA_4 release per minute per unit enzyme (V_0/E_0) were calculated based on the 210 nm peak area of GA_4. For the reactions varying the concentrations of GA_4-GE donor, sodium azide was fixed at 50, 100, 200 and 400 mM, and reactions without sodium azide were used as controls.

CHAPTER 3. Results

3.1 Syntheses of GA-glucosyl conjugates

3.1.1 Synthesis of the glucosyl ester of GA_4

Acetylated and deacetylated GA_4-GE esters (Figure 3.1) were synthesized as described in section 2.2.1. The acetylated and deacetylated GA_4-GE were obtained with 43.7% and 40.5% yields, respectively. The synthesized acetylated and deacetylated GA_4-GE structures were confirmed by NMR spectra (Figures 3.2 & 3.3) on a 300 MHz NMR spectrometer with a Varian 300 ID/PFG probe at a frequency of 299.986 MHz (Unity INOVA, Varian, USA). Deuterated chloroform ($CDCl_3$) and dimethyl sulfoxide-d_6 (DMSO-d_6) were used as solvents for acetylated and deacetylated GA_4-GE, respectively. The ^1H NMR was consistent with the published data for GA_4-GE [Hiraga et al., 1974].

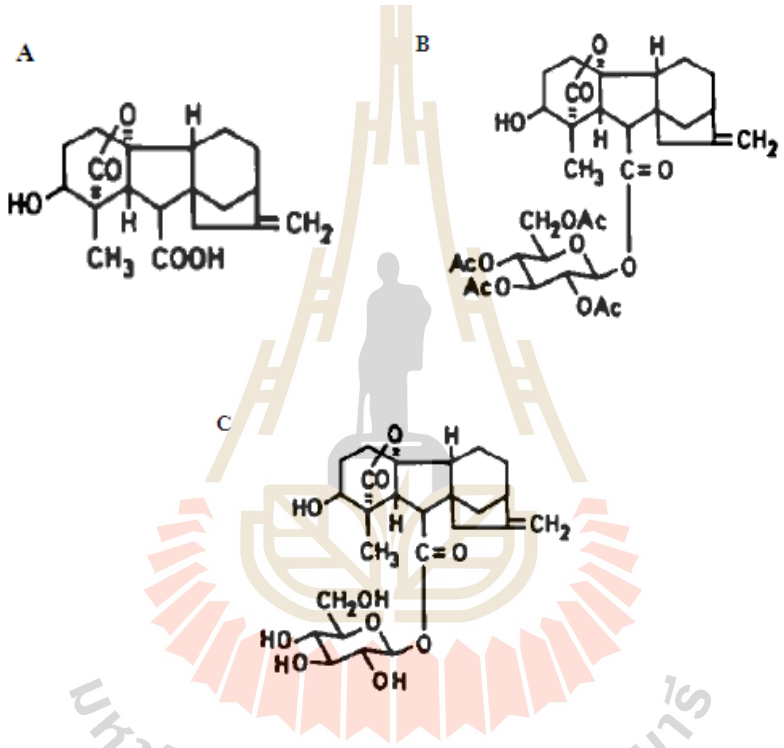

Figure 3.1 The structures of the gibberellin GA_4 and its derivatives. **A**. GA_4, $C_{19}H_{24}O_5$, molecular weight 332.39; **B**. Acetylated GA_4-GE, $C_{33}H_{42}O_{14}$, molecular weight 662.68; **C**. GA_4-GE, $C_{25}H_{34}O_{10}$, molecular weight 494.53.

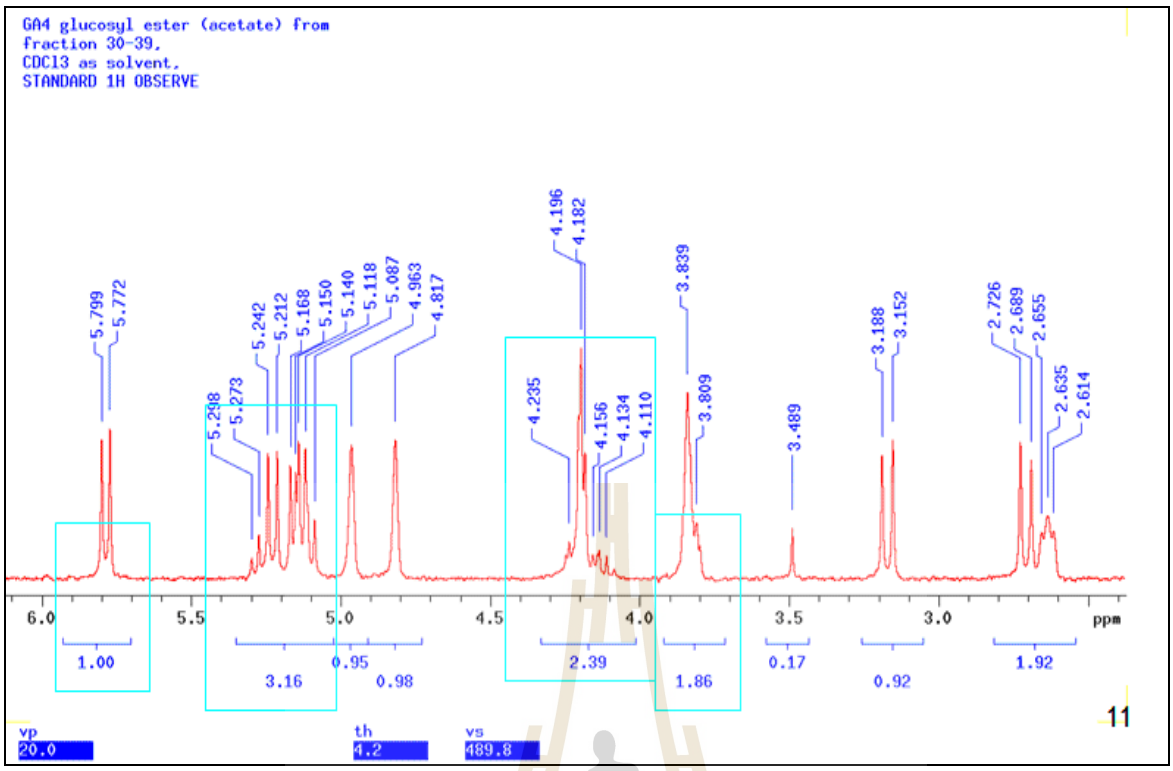

Figure 3.2 Expanded view of the ^{1}H NMR spectrum of acetylated GA_4-GE in $CDCl_3$. The 7 protons on the glucosyl ring are boxed in rectangles (one proton from GA_4 was included at position δ3.839). The coupling constant of the H1 peak at δ5.786 ($J_{2,1}$=8.1 Hz) confirmed that the acetylated GA_4-GE had a β-configuration.

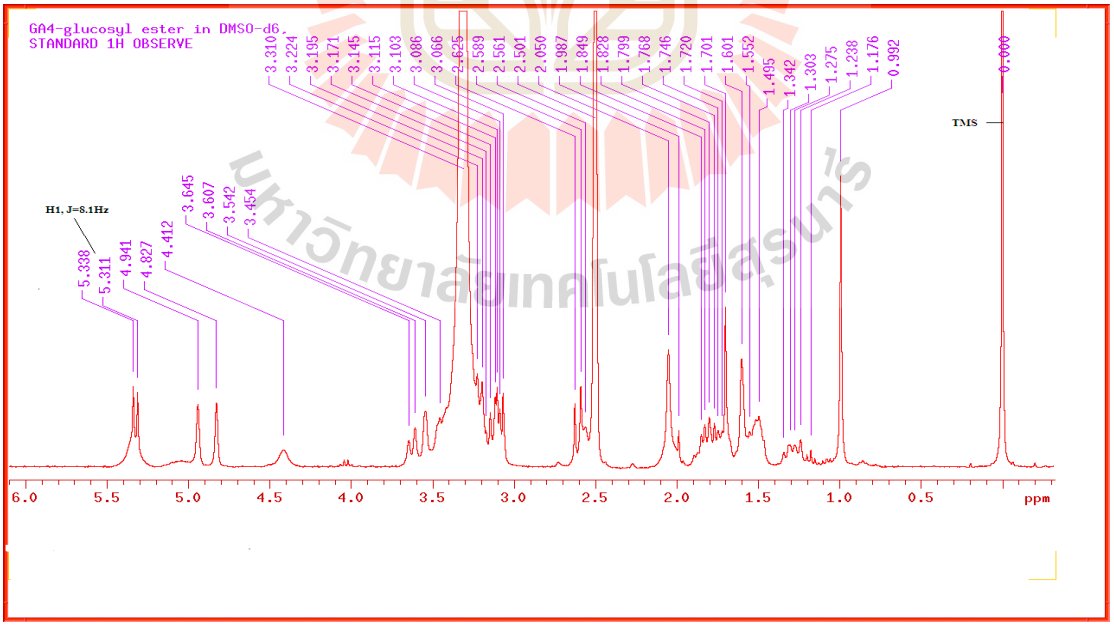

Figure 3.3 ^{1}H NMR spectrum of GA_4-GE in DMSO-d6 with TMS as reference standard. δ5.32 was assigned as H1, d, $J_{2,1}$=8.1 Hz, which confirmed that the GA_4-GE had a β-configuration.

The identity of the deacetylated GA_4-GE was also confirmed from its mass spectrum (Figure 3.4). In the positive mode, we detected $[M+Na]^+$ at m/z 517.1, $[M+H-H_2O]^+$ at m/z 477.2, and $[M+H-Glc]^+$ at m/z 315.5.

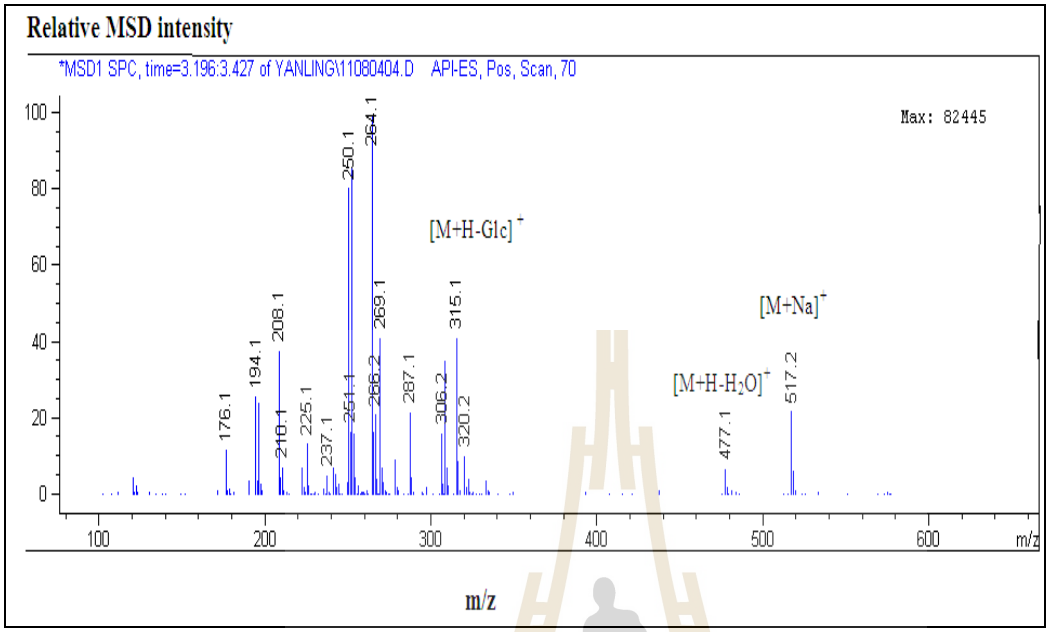

Figure 3.4 The mass spectrum of GA_4-GE in the positive mode. $[M+Na]^+$, $[M+H-H_2O]^+$ and $[M+H-Glc]^+$ are marked on the mass spectrum.

3.1.2 Synthesis of the glucosyl ester of GA_3

The structures of acetylated and deacetylated GA_3-GE esters (Figure 3.5) were confirmed by NMR and mass spectrometry. In the ^1H NMR spectra of the acetylated GA_3-GE (Figure 3.6), the peak for the H1 proton on the glucosyl ring was located at 5.81 ppm, with a coupling constant of 7.8 Hz, which confirmed that the acetylated GA_3-GE had a β-configuration.

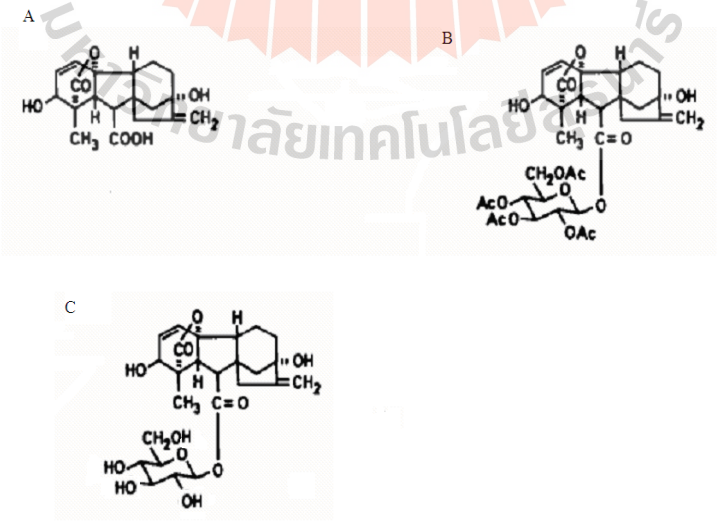

Figure 3.5 The structures of the gibberellin GA_3 and its derivatives. **A**. GA_3, $C_{19}H_{22}O_6$, molecular weight 346.37; **B**. Acetylated GA_3-GE, $C_{33}H_{40}O_{15}$, molecular weight 676.36; **C**. GA_3-GE, $C_{25}H_{32}O_{11}$, molecular weight 508.51.

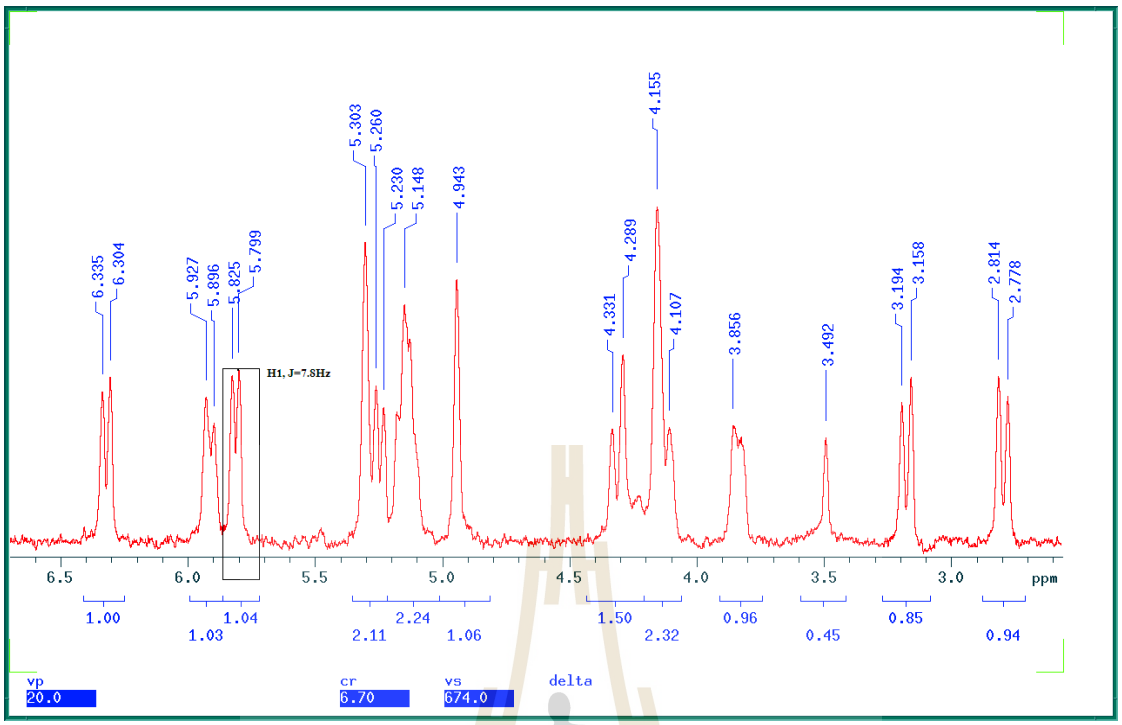

Figure 3.6 The expanded ^{1}H NMR spectra of the acetylated GA_3-GE in $CDCl_3$. TMS was the reference standard. The peak for the anomeric H1 proton on the glucosyl ring is enclosed in the box.

The deacetylated GA_3-GE ester was confirmed from its mass spectrum (Figure 3.7). In the negative mode, $[M+^{35}Cl]^-$ at *m/z* 543.4, $[M+^{37}Cl]^-$ at *m/z* 545.2 and $[M+HCO_2]^-$ at *m/z* 553.3 were detected. The β-configuration of the GA_3-GE was confirmed from the peak for the anomeric H1 proton on the glucosyl ring, which was located at 5.55 ppm, with $J_{1,2}$=8.4 Hz (Figure 3.8). The ^{1}H NMR of GA_3-GE was also consistent with the published data (Hiraga et al., 1974).

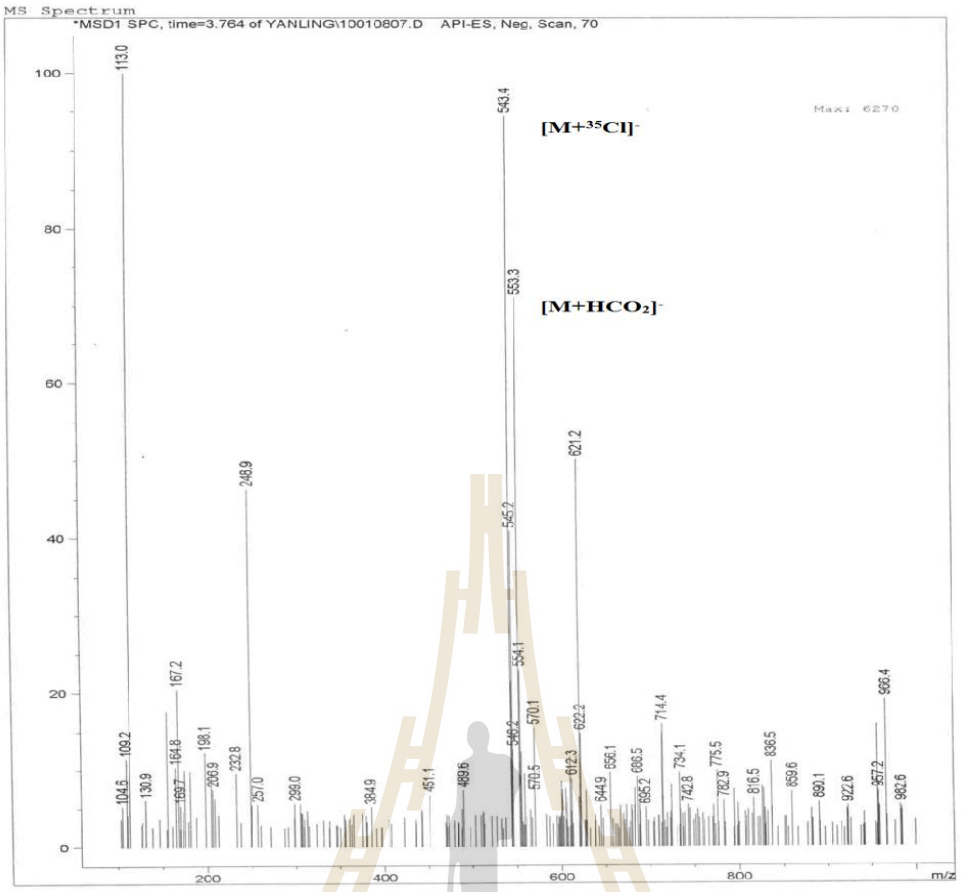

Figure 3.7 The mass spectrum of GA_3-GE in the negative mode. Peaks for $[M+ {}^{35}Cl]^-$ at *m/z* 543.4, $[M+ {}^{37}Cl]^-$ at *m/z* 545.2 and $[M+HCO_2]^-$ at *m/z* 553.3 are found in the mass spectrum.

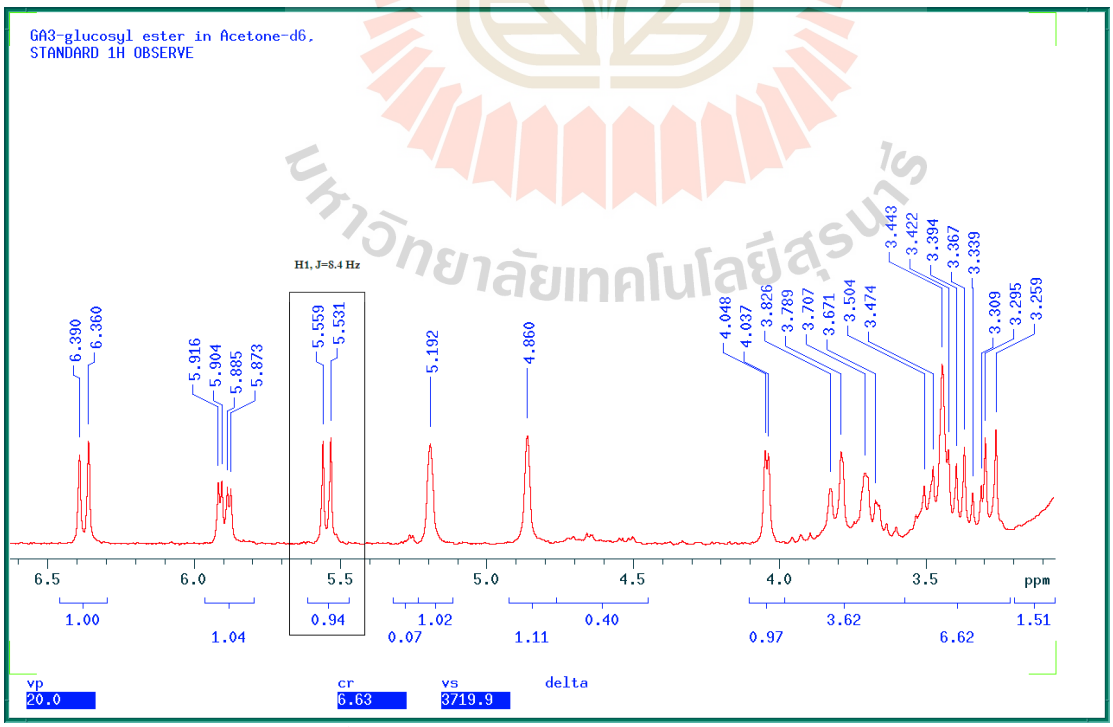

Figure 3.8 The expanded ^1H NMR spectra of the GA_3-GE in acetone-d6. The doublet for the H1 proton on the glucosyl ring is boxed in the rectangle.

3.2 Extraction, purification and characterization of GA_4-glucosyl ester β-glucosidase from rice

Ten kilograms of 10-day rice seedling shoots and leaves were extracted and the crude proteins were purified with seven purification steps (Table 3.1 and Figure 3.9). *p*NPGlc and GA_4-GE were used as substrates to test the β-D-glucosidase activities. The protein fractions with high hydrolysis activities toward GA_4-GE were pooled, concentrated and used for the next purification step. Finally, 0.15 mg of protein was obtained after seven steps of purification. This protein included two major bands that were seen in 10% SDS-PAGE and identified.

Table 3.1 Summary of purification of β-glucosidase in rice
Yield was calculated based on the amount of the protein obtained in each purification steps.

Purification step	Total activity (µmol/min.)		Protein (mg)	Specific activity (µmol/min.mg)		Purification fold		Yield (%)
	*p*NP-Glc	GA_4-GE		*p*NP-Glc	GA_4-GE	*p*NP-Glc	GA_4-GE	
1. Crude extract	5,710	11.0	27,200	0.21	$4.03*10^{-4}$	1	1	100
2. After $(NH_4)_2SO_4$ precipitation & dialysis	2,220	6.17	12,000	0.18	$5.12*10^{-4}$	0.9	1.3	44.3
3. CM-Sepharose column	1,470	4.22	1,215	1.21	$3.47*10^{-3}$	5.8	8.6	4.47
4. Con A-Sepharose column	281	1.57	140	2.01	$1.12*10^{-2}$	9.6	27.8	0.51
5. Sephadex 75 column	157	1.50	57	2.73	$2.62*10^{-2}$	13	65	0.21
6. SP xl column	30.4	0.36	6	5.07	$6.0*10^{-2}$	24	149	0.02
7. Octyl column	3.07	0.05	0.63	4.88	$8.1*10^{-2}$	23	201	0.002
8. Superdex 200 column	1.63	0.02	0.15	10.9	0.136	52	337	$5.5*10^{-4}$

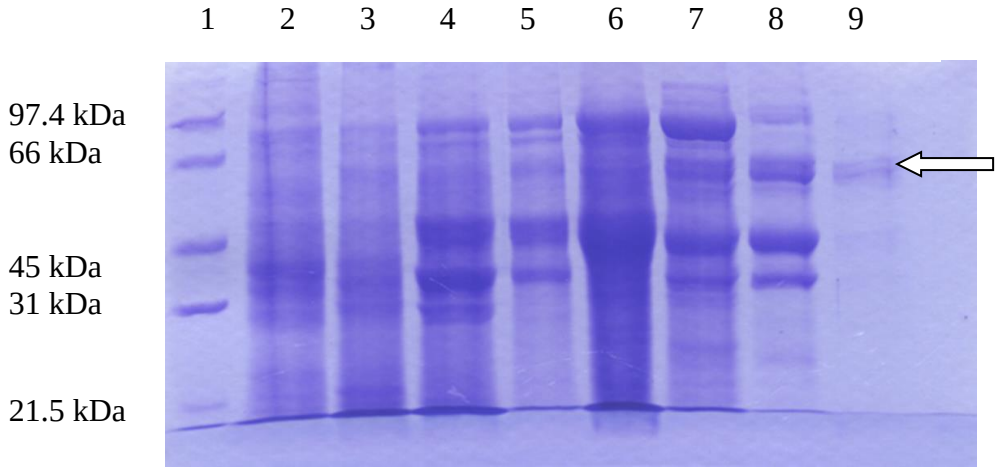

Figure 3.9 10% SDS-PAGE analysis of β-glucosidase purification from rice seedlings. Lane 1, protein marker; lane 2, crude extract; lane 3, crude extract after dialysis; lane 4, protein after CM column chromatography; lane 5, protein after Con A column chromatography; lane 6, protein after S75 column chromatography; lane 7, protein after SP column chromatography; lane 8, protein after octyl column chromatography; lane 9, protein after S200 column chromatography.

3.2.1 Purification of β-glucosidase from rice by ion exchange chromatography with a CM-Sepharose column

The crude protein after $(NH_4)_2SO_4$ precipitation & dialysis with McIlvaine buffer was fractionated with a CM-Sepharose column (20 ml), which was equilibrated with 4-fold diluted McIlvaine buffer, pH 5, and eluted with a linear gradient of 0-1.0 M NaCl in this buffer (Figure 3.10). Two peaks were detected in the bound proteins, and their activities were tested with *p*NPGlc and GA_4-GE as described in Chapter II (Figure 3.11).

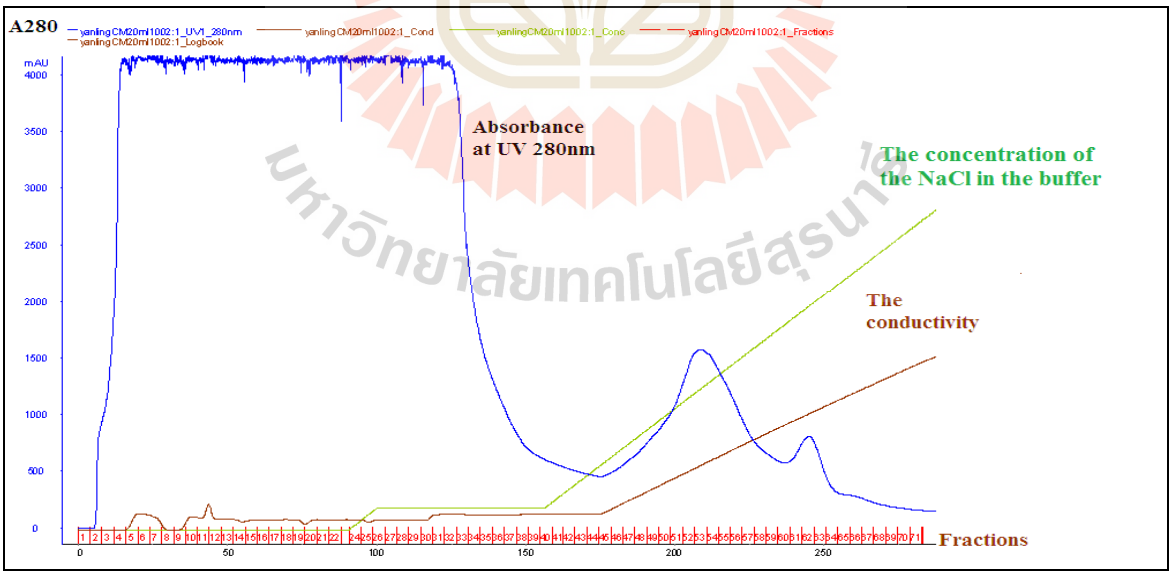

Figure 3.10 Protein elution profile from CM-Sepharose column**.** The unbound proteins were washed out with 4-fold diluted McIlvaine buffer, pH 5 (buffer A), and the bound proteins were eluted with a linear gradient of 0-1.0 M sodium chloride (NaCl) in buffer A at a flow rate of 2.0 ml/min. The fraction volume was 4 ml.

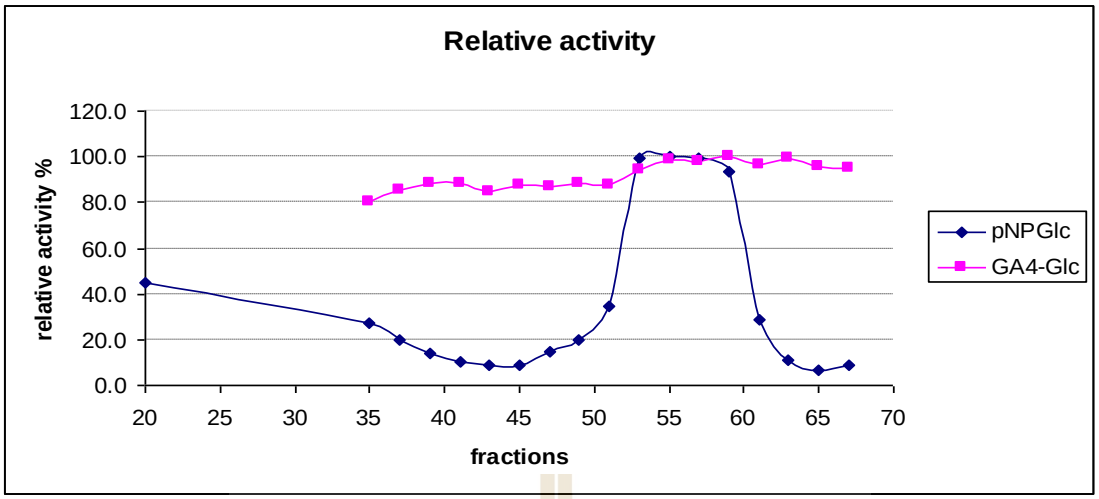

Figure 3.11 Relative β-glucosidase activities of eluting fraction from CM-Sepharose column with *p*NPGlc and GA_4-GE as substrates. Relative activity (%) was based on the absorbance at 405 nm in the respective assays.

The activities of fractions from the CM-Sepharose column for hydrolysis of *p*NPGlc showed that there were two peaks in the bound proteins, peak 1 had higher hydrolysis activity than peak 2 and the unbound proteins peak. For the activities with GA_4-GE, it was too low to conclude which peak had higher activity toward GA_4-GE. So, based on the *p*NPGlc hydrolysis activity, two peaks from bound proteins were pooled, concentrated by $(NH_4)_2SO_4$ precipitation, dialyzed, and used for next step of purification.

3.2.2 Purification of β-glucosidase from rice by affinity chromatography with a ConA-Sepharose column

The fractions from ConA-Sepharose column were divided into four groups: flow-through 1, flow-through 2, eluate 1 and eluate 2. Eluate 1 & 2 showed higher activities than flow-through 1 & 2 for hydrolyzing GA_4-GE (Figure 3.12), so these two fractions were pooled and used for further purification.

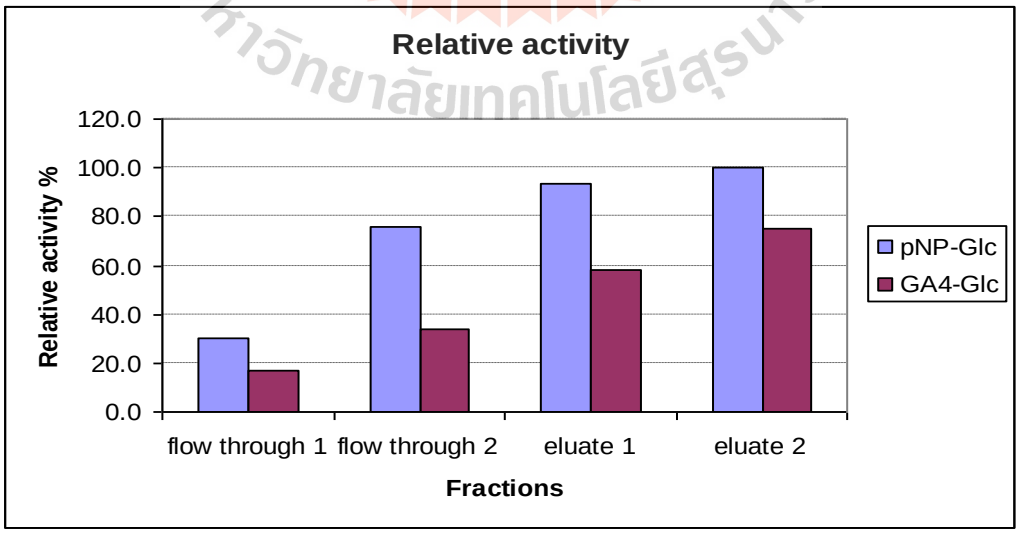

Figure 3.12 Relative activities of flow-through and eluting fractions from the ConA-Sepharose column. The relative activities were calculated based on the specific activities.

3.2.3 Purification of β-glucosidase in rice by gel filtration chromatography through a Superdex S75 column

The active fractions from the ConA-Sepharose column were concentrated and purified with a S75 gel filtration column (Figure 3.13). The eluting fractions were tested for hydrolysis of *p*NPGlc and GA_4-GE (Figure 3.14). The fractions 62-74 showed the highest activities to hydrolyze GA_4-GE. These fractions were run on SDS-PAGE to check their protein compositions (Figure 3.15), and then pooled for further purification.

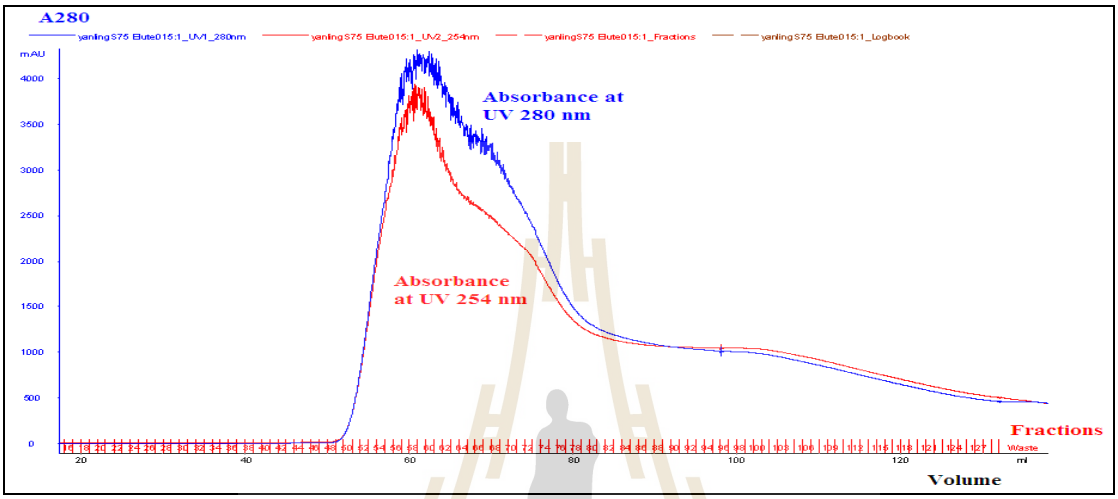

Figure 3.13 Protein elution profile of the Superdex S75 column. The protein was eluted at a flow rate of 1.0 ml/min, and the fraction volume was 1 ml.

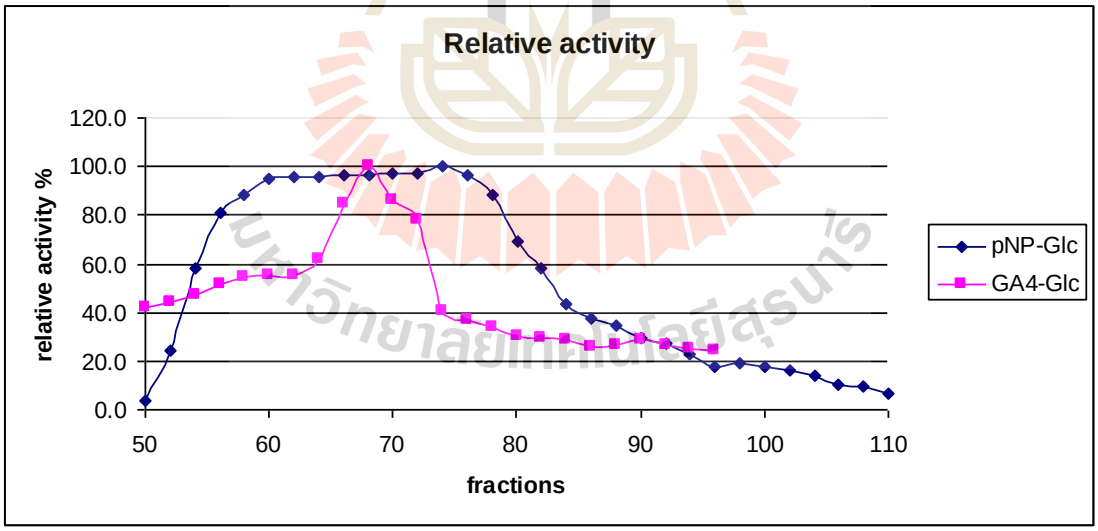

Figure 3.14 Activities of eluting fractions from S75 column with *p*NPGlc and GA_4-GE. ♦Hydrolysis activity against *p*NPGlc, ■Hydrolysis activity against GA_4-GE. Relative activity (%) was calculated based on the absorbance at 405 nm obtained in the respective assays.

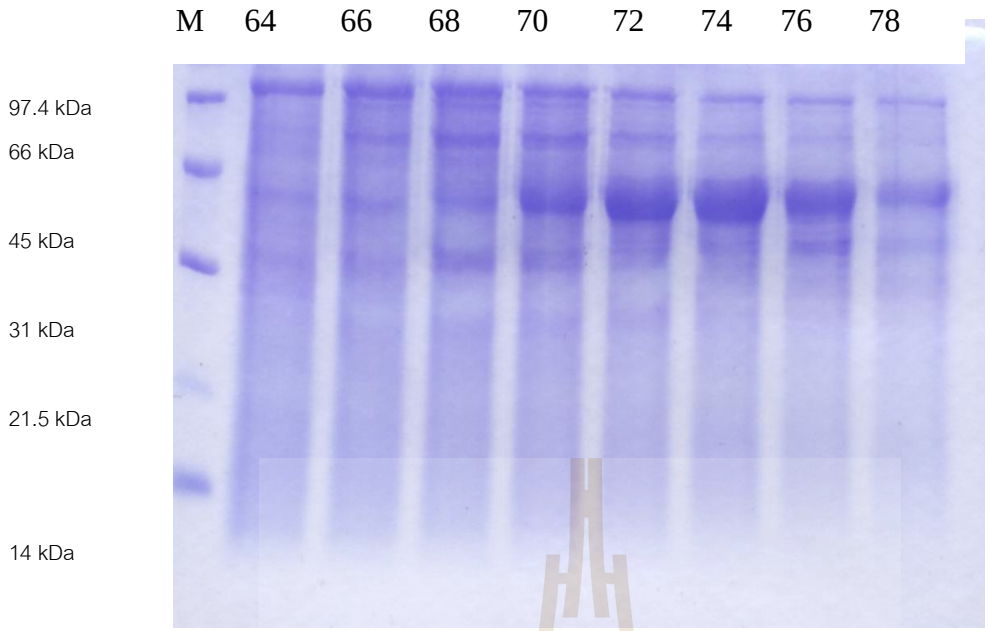

Figure 3.15 SDS-PAGE analyses of fractions after S75 column purification. Lane M, marker; lane 64-78 represented fractions 64-78.

3.2.4 Purification of β-glucosidase from rice by cation exchange chromatography with an SP XL column

The concentrated protein pool from the S-75 gel filtration column was purified with a HiTrap SP XL column (Figure 3.16). The eluting fractions were tested for hydrolysis of *p*NPGlc and GA_4-GE. Figure 3.17 shows that fractions 1-2, 8-13 could hydrolyze *p*NPGlc, but only fractions 8-10 showed relatively high hydrolysis activities with GA_4-GE. Since the fractions still had multiple bands on SDS-PAGE (Figure 3.18), fractions 8-10 were pooled and used for further purification.

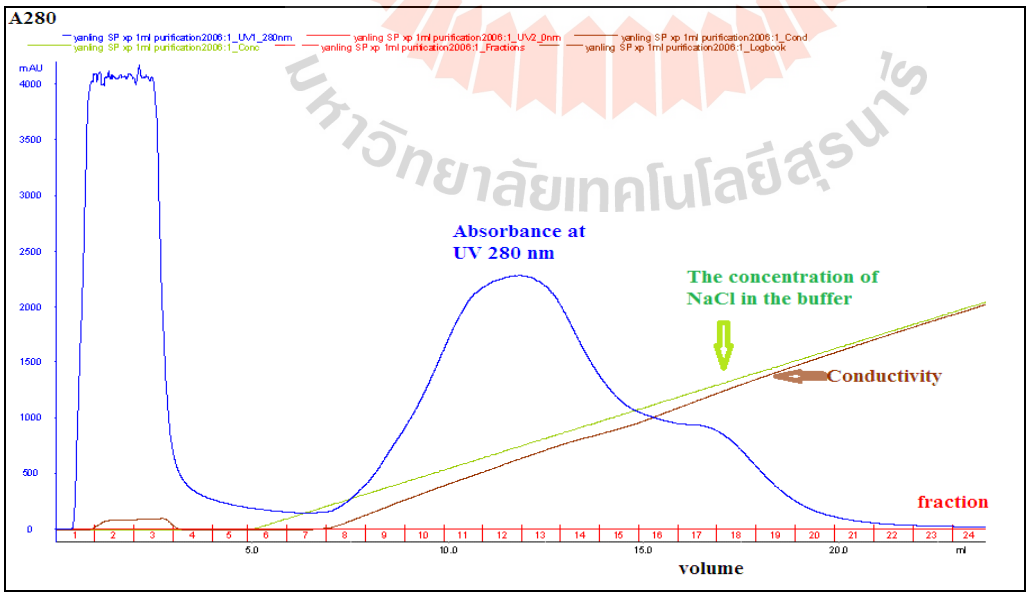

Figure 3.16 Protein elution profile of SP column. The column was eluted with a linear gradient of 0-1.0 M NaCl in 4-fold diluted McIlvaine buffer, pH 5, at a flow rate of 1.0 ml/min. The fraction volume was 1.0 ml.

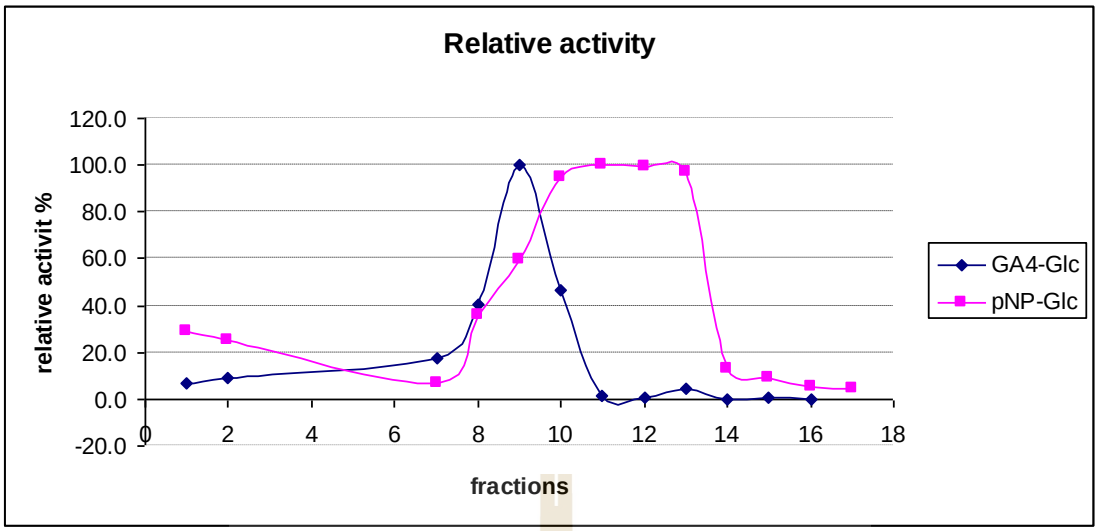

Figure 3.17 β-Glucosidase activities of eluting fractions from SP column with *p*NPGlc and GA_4-GE (denoted GA4-Glc) substrates. ■ Hydrolysis activity with *p*NPGlc, ♦ Hydrolysis activity with GA_4-GE. The relative activities (%) were calculated based on A_{405}.

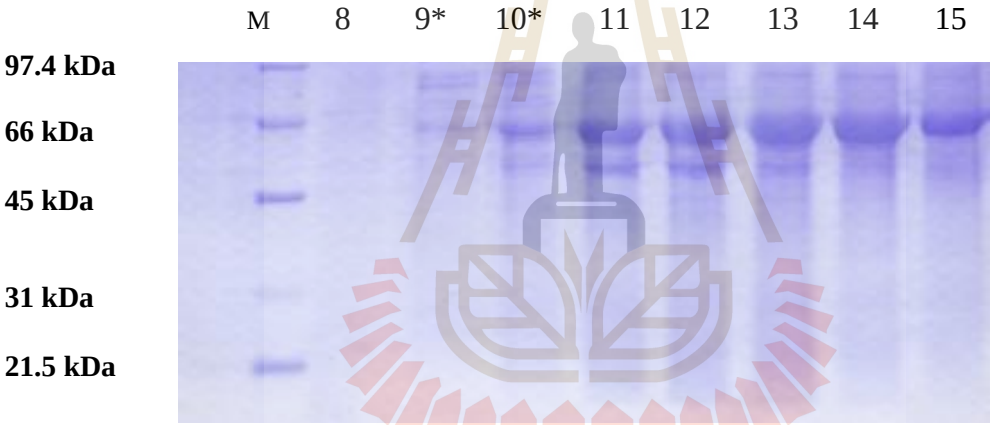

Figure 3.18 SDS-PAGE analysis of fractions containing high activity against GA_4-GE after SP column chromatography. Lane M, marker; lanes 8-15 represented fractions 8-15. Two bands at size around 60 kDa appeared in fraction 9 and 10, and these two fractions had the highest hydrolysis activity against GA_4-GE.

3.2.5 Purification of β-glucosidase by hydrophobic interaction chromatography over an Octyl Sepharose 4 column

The β-glucosidase pool obtained from the SP column purification was purified with hydrophobic Octyl-Sepharose 4 column. Most of proteins were bound to the column and eluted at reduced $(NH_4)_2SO_4$ concentration in phosphate buffer (Figure 3.19). The buffer of the collected fractions was exchanged twice with 4 times diluted McIlvaine buffer, pH 5, to remove $(NH_4)_2SO_4$; and then the fractions were tested for hydrolysis of GA_4-GE (Figure 3.20) and aliquots were analyzed by SDS-PAGE to check protein components (Figure 3.21). Fractions 27-33 were found to have higher hydrolysis activities than other fractions. Three groups of bands were mainly seen in the fractions 27-33, at sizes of approximately 60 kDa, 45 kDa and 33 kDa. The bands at 45 kDa and 33 kDa were also evident in the fractions 11, 12 and 13 of the SP column purification (Figure 3.18) in high amounts, but these fractions did not have activities for hydrolysis of GA_4-GE. Only two major bands at approximately 60 kDa

size were highly correlated with the activity. So fractions 27-33 were pooled, concentrated and purified further by gel filtration chromatography.

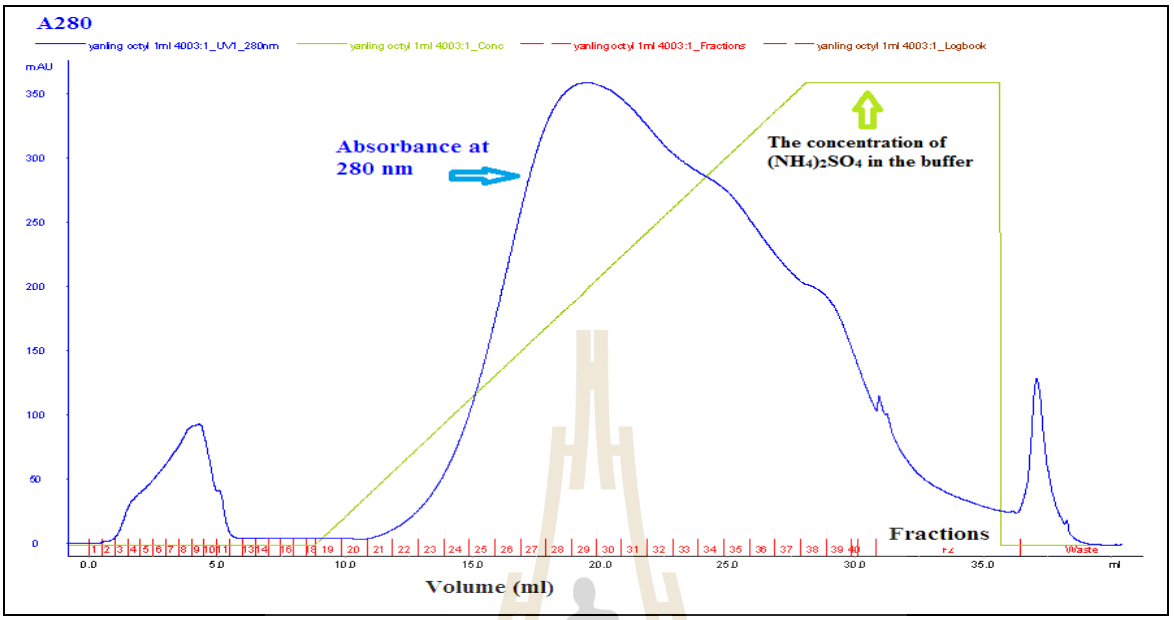

Figure 3.19 Protein elution profile of octyl Sepharose 4 column. The protein was eluted with a linear gradient of 1.7-0 M of $(NH_4)_2SO_4$ in 50 mM phosphate, pH 7, buffer. The elution fraction volume was 1.0 ml each.

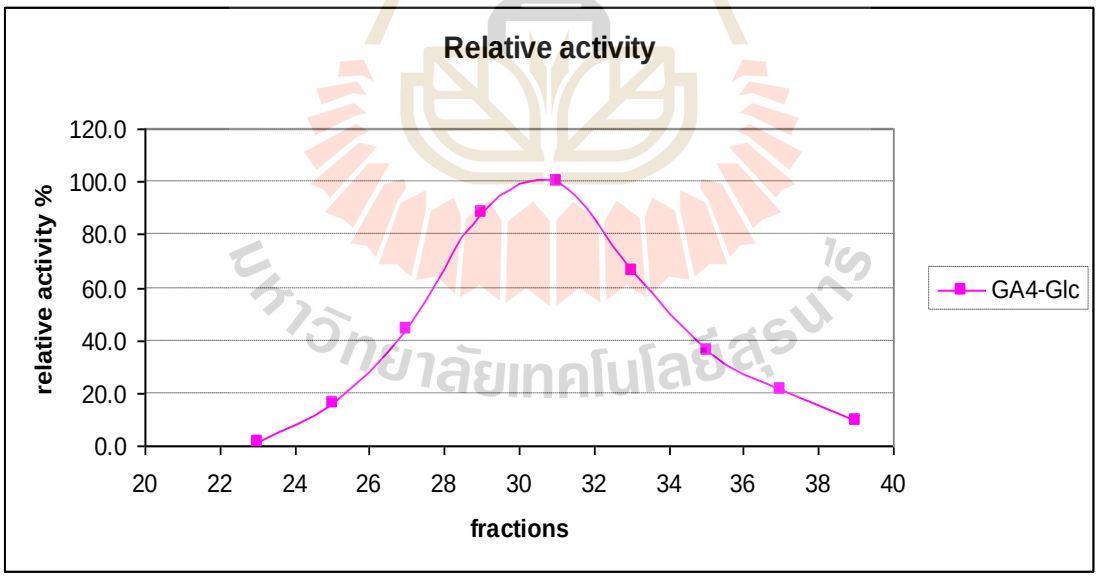

Figure 3.20 Activities of fractions eluted from the octyl Sepharose column for hydrolysis of GA_4-GE. The relative activities (%) were calculated based on A_{405}.

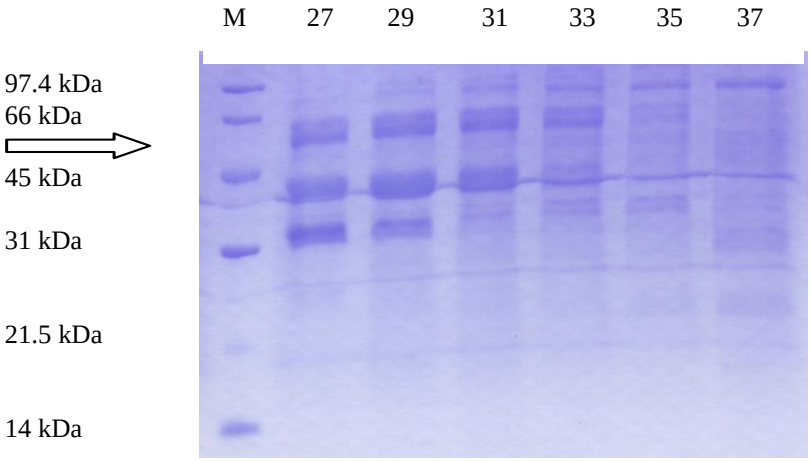

Figure 3.21 SDS-PAGE analysis of fractions after octyl hydrophobic interaction chromatography. Lane M, marker; lanes 27-37 represent the corresponding fractions 27-37.

3.2.6 Purification of β-glucosidase with an S200 gel filtration column

The active fraction pool from the Octyl column purification was further purified with gel filtration over a Superdex S200 column (Figure 3.22). The buffer in the eluting fractions was exchanged to 4-fold diluted McIlvaine buffer, pH 5, with an ultra centrifugal filter before activity tests. Fractions 29 and 30 were found to have the highest hydrolysis activities with GA_4-GE (Figure 3.23). In the 10% SDS-PAGE, two bands with size at approximately 60 kDa were evident as major bands in the fractions 29 and 30 (Figure 3.24).

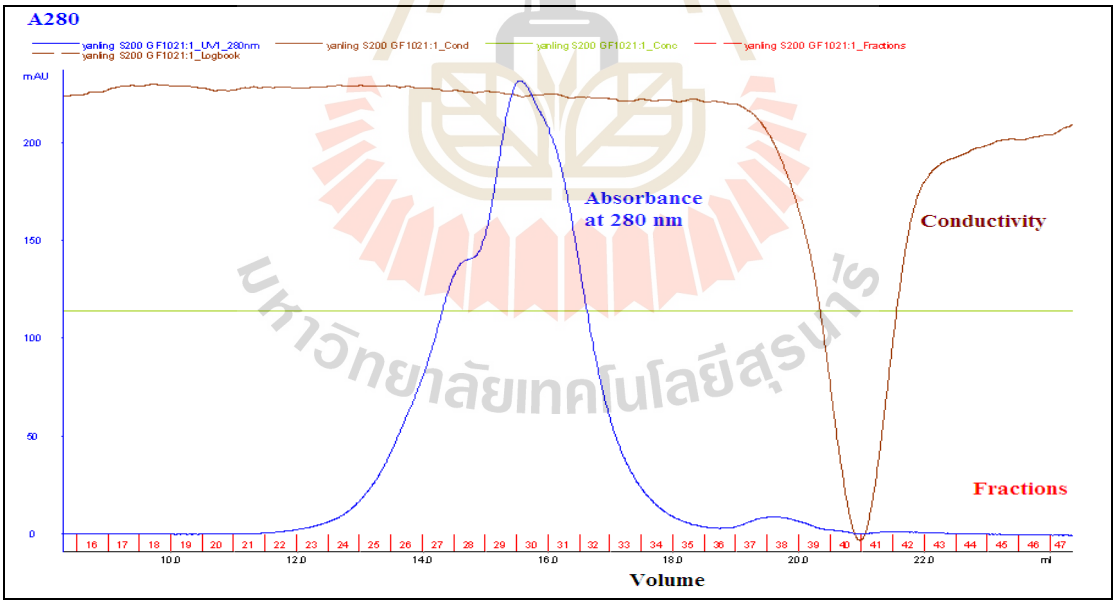

Figure 3.22 Protein elution profile of Superdex S200 gel filtration column. The protein was eluted at a flow rate of 0.5 ml/min; and the fraction volume was 0.5 ml.

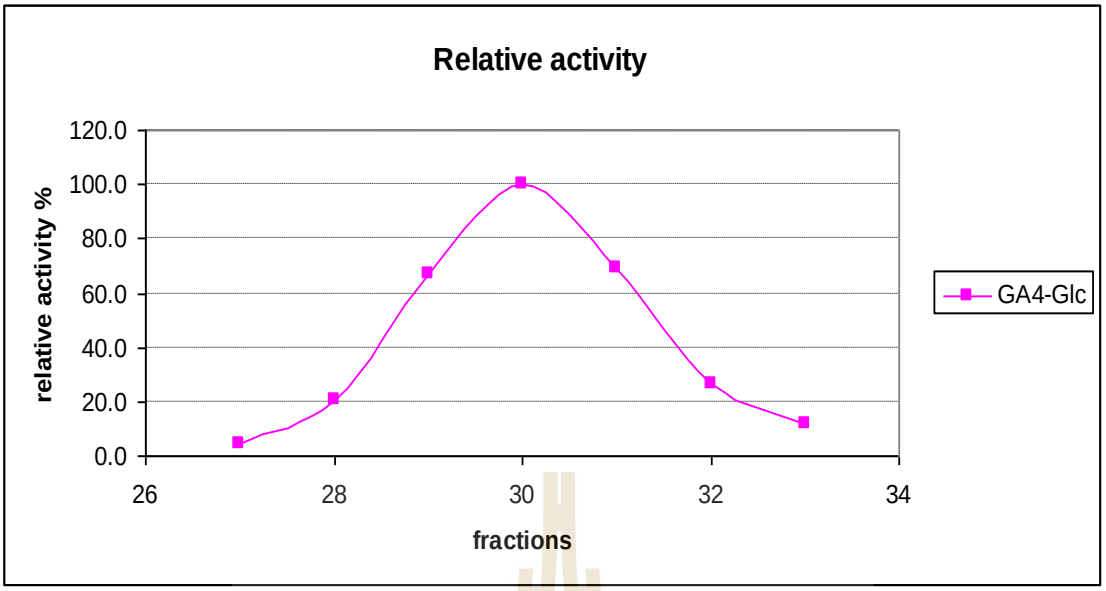

Figure 3.23 Activities of eluting fractions from Superdex S200 gel filtration chromatography for hydrolysis of GA_4-GE. The relative activities (%) were calculated based on A_{405}.

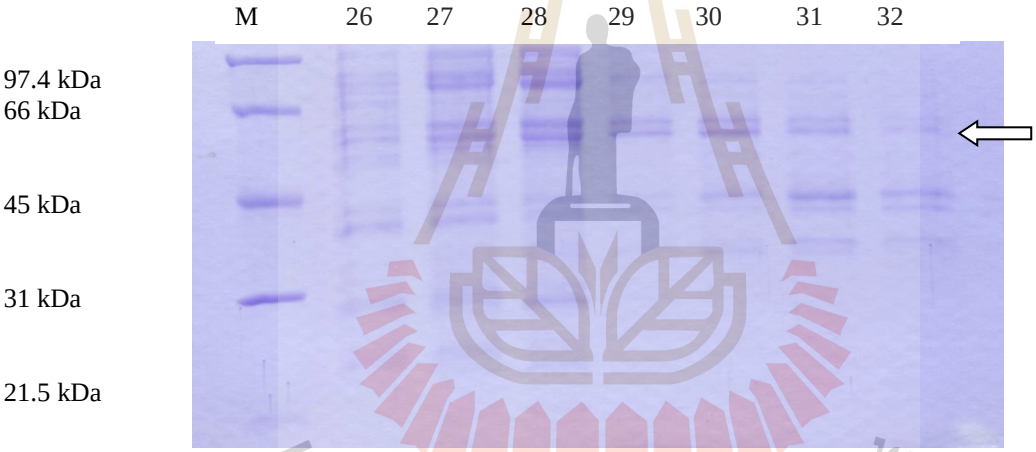

Figure 3.24 SDS-PAGE analysis of fractions after Superdex S200 gel filtration chromatography. Lane M, marker; lanes 26-32 represent fractions 26-32.

3.2.7 Identification of β-glucosidase with LC-MS

The two major protein bands from Superdex S200 column fraction 29 were identified by LCMS of tryptic peptides generated from their SDS-PAGE gel bands (Figure 3.25). The peptide mass results were used in a MASCOT search of the Genbank *non-redundant (*nr) protein database. The peptide masses from the lower band matched the protein product from the Os04g0474900 gene locus [*Oryza sativa* Japonica Group], which corresponds to Os4BGlu13 β-glucosidase, from glycoside hydrolase family 1 (Table 3.2). The peptide masses from the upper band matched a hypothetical protein OsI_23311 [*Oryza sativa* Indica Group] (Table 3.3).

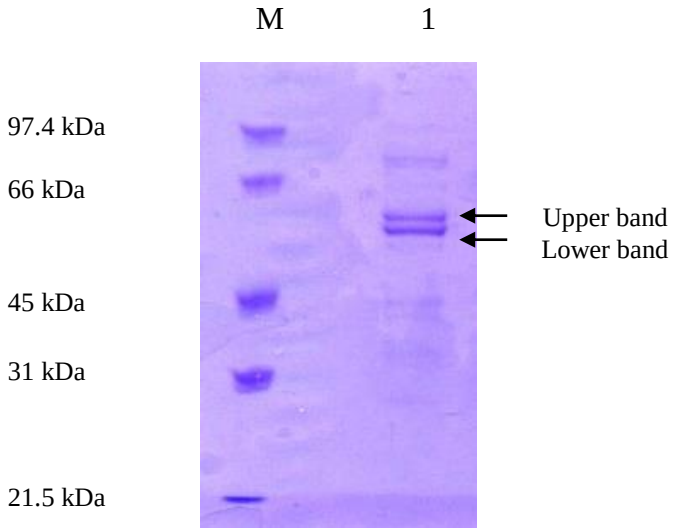

Figure 3.25 8% SDS-PAGE of fraction 29 in preparation for tryptic digestion and mass spectrometric analysis of derived peptides. Lane M, protein marker; lane 1, fraction 29. The two major bands were excised separately and submitted for tryptic digestion followed by LC-MS analysis.

Table 3.2 MASCOT search results for lower band

Protein View: **gi|115458942**

Os04g0474900 [*Oryza sativa* Japonica Group]

Database: NCBInr
Score: 1006
Nominal mass (M_r): 57,386
Calculated pI: 6.44
Taxonomy: *Oryza sativa* Japonica Group

Search parameters

Enzyme: Trypsin: cuts C-term side of K or R, unless next residue is P.
Fixed modifications: Carbamidomethyl (C)
Variable modifications: Oxidation (M)

Protein sequence coverage: 49%

Matched peptides are shown in ***bold***.

1	MAAAGEVVML	GGILLPLLLV	VAVSGEPPPI	SRRSFPEGFI	FGTASSSYQY
51	EGGAREGGRG	PSIWDTFTHQ	HPDKIADKSN	GDVAADSYHL	YKEDVRIMKD
101	MGVDAYRFSI	SWTRILPNGS	LSGGINREGI	SYYNNLINEL	LLKGVQPFVT
151	LFHWDSPQAL	EDKYNGFLSP	NIINDYKEYA	ETCFKEFGDR	VKHWITFNEP
201	LSFCVAGYAS	GGMFAPGRCS	PWEGNCSAGD	SGREPYTACH	HQLLAHAETV
251	RLYKEKYQVL	QKGKIGITLV	SNWFVPFSRS	KSNIDAARRA	LDFMLGWFMD
301	PLIRGEYPLS	MRELVRNRLP	QFTKEQSELI	KGSFDFIGLN	YYTSNYAGSL
351	PPSNGLNNSY	STDARANLTA	VRNGIPIGPQ	AASPWLYIYP	QGFRELVLYV
401	KENYGNPTIY	ITENGVDEFN	NKTLPLQEAL	KDDTRIDYYH	KHLLSLLSAI

451	RDGANVKGYF	AWSLLDNFEW	SNGYTVRFGI	NFVDYNDGAK	RYPKMSAHWF
501	KEFLQK				

Table 3.3 MASCOT search results for upper band

Protein View:	**gi|125555680** hypothetical protein OsI_23311 [*Oryza sativa* Indica Group]
Database:	NCBInr
Score:	851
Nominal mass (M_r):	57,128
Calculated pI:	8.67
Taxonomy:	*Oryza sativa* Indica Group

Search parameters

Enzyme:	Trypsin: cuts C-term side of K or R, unless next residue is P.
Fixed modifications:	Carbamidomethyl (C)
Variable modifications:	Oxidation (M)

Protein sequence coverage: 35%

Matched peptides shown in ***bold***.

1	MAAAPMSFAF	TLLAACISFL	HHAPAAAAAA	PANQTAGFLD	CLAASLPAGV
51	VYTHASR**SYQ**	**SVLESSIKNL**	**LFDTPATPTP**	**VAVVEATDAS**	**HVQAAVR**CGV
101	GHGVSVRSRS	GGHDYEGLSY	RSLDAAR**AFA**	**VVDMAGGALR**	AVRVDVRGRA
151	AWVGSGATLG	EVYYAIANKT	SRLGFPGSVG	PTVGVGGFLS	GGGFGLMLRK
201	**HGLASDHVLD**	**ATMVDAK**GRL	LDR**AAMGEDL**	**FWAIRGGGGG**	**NFGIVLSWK**L
251	RLVPVPATVT	VFTVHRSRNQ	SATDLLAKWQ	R**VAPSLPSDA**	**FLRVVVQNQN**
301	**AQFESLYLGT**	**R**AGLVAAMAD	AFPELNVTAS	DCIEMTWVQS	VLYFAFYGTG
351	KPPEMLLDRG	TGRPDRYFKA	KSDYVQEPMP	SQVWETTWSW	LLK**DGAGLLI**
401	**LDPYGGEMAR**	VAPAATPFPH	RQALYNIQYY	GFWSESGEAA	AAKHMGWIR**G**
451	**VYGEMEPYVS**	**K**NPRGAYVNY	R**DLDLGVNDD**	**GGGVAR**ARYE	KATVWGRAYF
501	KANFERLAAV	K**AKVDPDNYF**	**KNEQSIPPLP**	**S**	

3.3 Screening of Rice GH1 enzymes for GA_4-glucosyl ester hydrolysis

Five rice GH1 enzymes that have been expressed in our lab were tested for the hydrolysis of *p*NPGlc and GA_4-GE according to the methods described in section 2.2.2.9. As shown in Table 3.4, Os3BGlu6 was found to have the highest hydrolysis activity to GA_4-GE among these enzymes. Although Os9BGlu31 had a higher ratio of activity toward GA_4-GE compared to *p*NPGlc (0.267 vs. 0.07 for Os3BGlu6), it is primarily a transglycosidase and has very low activity toward both substrates.

Table 3.4 GA_4-GE hydrolysis by recombinantly expressed rice GH1 enzymes

Enzyme	Activity toward GA_4-GE (µmol Glc released/min/mg)	Activity toward *p*NPGlc (µmol *p*NP released/min/mg)	Ratio of activity toward GA_4-GE/*p*NPGlc
Os3BGlu6	0.185	2.6	0.07
Os3BGlu7 (BGlu1)	0.02	4.0	0.005
Os4BGlu12	0.035	130	0.003
Os4BGlu18	N.D.	0.94	-
Os9BGlu31	0.02*	0.075*	0.267

N.D. means not detectable.

* Activity is primarily transglycosylation, rather than hydrolysis (Luang et al., 2013).

3.4 Recombinant Expression and Purification of the Mutants of Os3BGlu6

The Os3BGlu6 protein expressed in *E. coli* was purified with 2 steps of IMAC, as described in section 2.2.5.3. The fractions were analyzed by 12% SDS-PAGE (Figure 3.26). After the 2nd IMAC, the protein appeared as a single band on the gel with about 90% purity.

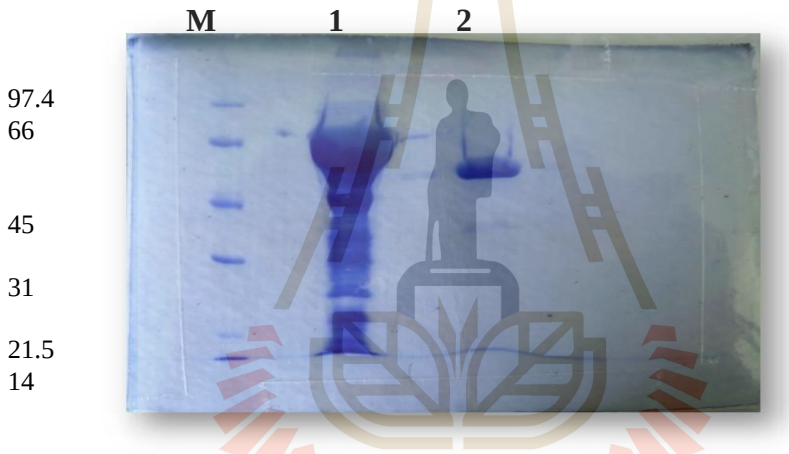

Figure 3.26 Comparison of Os3BGlu6 after 1st and 2nd IMAC purifications. Lane M, protein marker; lane 1, protein after 1st IMAC; lane 2, protein after 2nd IMAC.

Os3BGlu6 M251N, E178Q and E178A were purified by the same method as its wild type, and the protein fractions were analyzed with SDS-PAGE and found to give similar results to wild type Os3BGlu6 (data not shown).

3.5 Characterization of Os3BGlu6 and Its Mutants

3.5.1 pH optimum for Os3BGlu6 and its mutants

The Os3BGlu6 wild type and M251N were found to have high *p*NPGlc hydrolysis activity between pH 4.0 and 5.0, with highest value at pH 4.5, while the activity quickly dropped above pH 5.5, with 50% of maximal activity at approximately pH 3.3 and 5.6 for wild type, and pH 3.5 and 5.5 for M251N (Figure 3.27). With GA_4-GE substrate, Os3BGlu6 wild type and M251N, E178Q and E178A mutants also showed highest hydrolytic activities at pH 4.5 (Figure 3.28). The activities of Os3BGlu6 E178Q and E178A dropped slowly above pH 6.0, compared to Os3BGlu6 wild type and M251N, when hydrolyzing GA_4-GE.

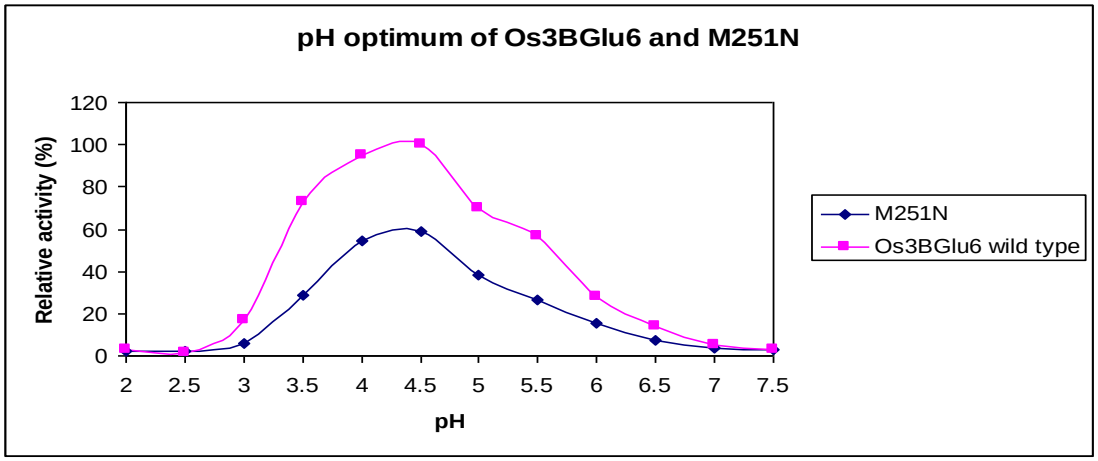

Figure 3.27 The pH-activity profiles of Os3BGlu6 and Os3BGlu6 M251N for hydrolysis of *p*NPGlc in universal buffer. One microgram of enzyme was incubating with 2 mM *p*NPGlc, in 80 µl of universal buffer (final volume was 100 µl), pH 2 to 11, in 0.5-pH-unit increments, at 30°C for 10 min. The reactions were stopped by adding 100 µl of 2 M Na_2CO_3. The relative activities were calculated based on A_{405}.

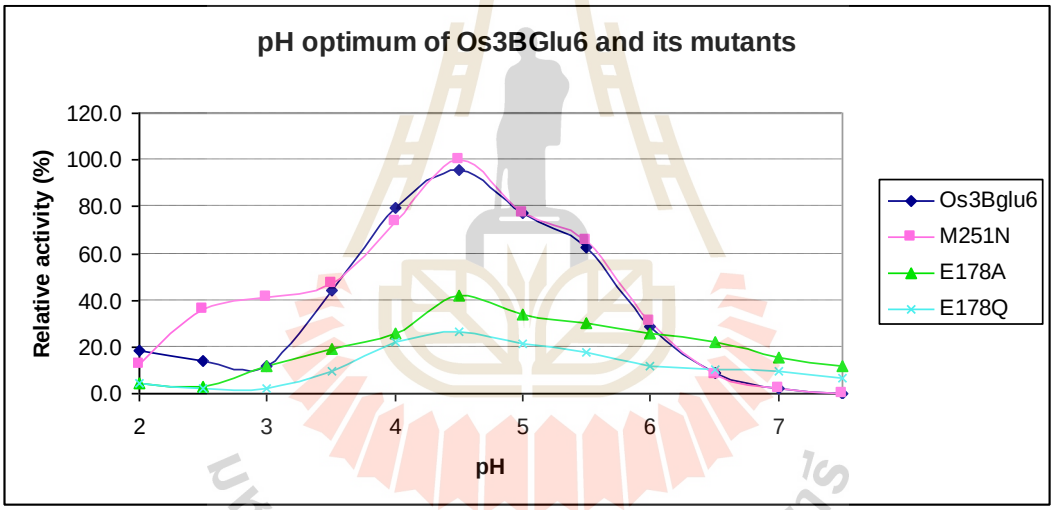

Figure 3.28 The pH-activity profiles of Os3BGlu6 and its mutants for hydrolysis of GA_4-GE in universal buffer. This figure showed 1 µg of Os3BGlu6 or Os3BGlu6 M251N, E178Q or E178A was incubated with 0.86 mM GA_4-GE in 80 mM universal buffer, pH 2 to 11, in 0.5-pH-unit increments, at 30° for 20 min. The relative activities are percentages of the highest activity observed, that of Os3BGlu6 M251N at pH 4.5.

3.5.2 Hydrolysis activities of Os3BGlu6 and its mutants

The structure of Os3BGlu6 wild type has been resolved by X-ray crystallography [Seshadri et al., 2009]. The residue M251 was found playing an important role in the substrate binding. The mutation of M251 to Asn resulted a 15-fold increase in hydrolysis efficiency with laminaribiose and 9 to 24-fold increases for cellooligosaccharides compared to wild type Os3BGlu6 [Sansenya et al., 2012]. In our study, the M251N mutant showed reduced hydrolytic activities for *p*NPGlc and GA_4-GE compared to its wild type. The relative activities of M251N were 51.6% for *p*NPGlc, 88.9% for GA_4-GE; the catalytic efficiencies (k_{cat}/K_m) for M251N were reduced to 2.6 from 6.2 $mM^{-1}s^{-1}$ for *p*NPGlc, 0.08 from 0.13 for GA_4-GE (Table 3.5) compared to its wild type. The mutation of the putative catalytic

acid/base glutamate residue at 178 to alanine (E178A) and glutamine (E178Q) led to complete loss of hydrolytic activity toward *p*NPGlc; and the relative activities to GA_4-GE were reduced to 22.2% and 12.5%, respectively, compared to its wild type. The mutation of the catalytic nucleophile glutamate residue at 394 to glutamine (E394Q) and aspartic acid (E394D) led to complete loss of hydrolytic activity toward both *p*NPGlc and GA_4-GE.

Table 3.5 Hydrolysis activities of Os3BGlu6 and its mutants.

Enzyme	***p*NP-β-D-glucoside**				**GA_4-GE**			
	Relative activity (%)	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m (mM^{-1} s^{-1})	Relative activity (%)	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m (mM^{-1} s^{-1})
Os3BGlu6	100	6.3 ± 0.4*	38.9 ± 0.9*	6.2*	100	5.8 ± 0.6	0.75 ± 0.04	0.13
Os3BGlu6-M251N	51.6	6.5 ± 0.7	15.4 ± 0.9	2.6	88.9	14.6 ± 2.0	1.2 ± 0.1	0.08
Os3BGlu6-E178Q	0.3	n.m.	n.m.	n.m.	12.5	0.09 ± 0.01	0.03 ± 0.001	0.33
Os3BGlu6-E178A	0.7	n.m.	n.m.	n.m.	22.2	3.7 ± 0.4	0.09 ± 0.005	0.03
Os3BGlu6-E394D	0.9	n.m.	n.m.	n.m.	1.4	n.m.	n.m.	n.m.
Os3BGlu6-E394Q	0.8	n.m.	n.m.	n.m.	0.6	n.m.	n.m.	n.m.

n.m.: not measured. *Data from Seshadri et al. [2009]. For the relative activity with *p*NPGlc, 0.5 µg of Os3BGlu6 wild type or M251N, 3 µg of E178A, E178Q, E394D or E394Q was incubating with 4 mM *p*NPGlc in NaOAc buffer (final volume was 50 µl), at 30°C for 10 min. For the relative activity with GA_4-GE, 1.0 µg of Os3BGlu6 wild type or M251N, 3 µg of E178A, E178Q, E394D or E394Q was incubating with 1.7 mM GA_4-GE in NaOAc buffer (final volume was 50 µl), at 30°C for 20 min. The relative activities were calculated based on A_{405} and the amount of the enzymes. For the kinetic activities, 50 mM NaOAc buffer was used for Os3BGlu6 wild type and M251N, for both substrates; but 50 mM MES buffer was selected for the E178Q and E178A mutants.

3.5.3 Identification of transglucosylation product with TLC, LC-MS and NMR

Os3BGlu6 hydrolyzed *p*NPGlc in MES buffer to *p*NP and glucose, and no transglucosylation products were formed when GA_4 and azide were added as acceptors. Neither Os3BGlu6 wild type nor E178Q could hydrolyze dNP2FGlc, and no transglucosylation product was formed when GA_4 was used as an acceptor to try to rescue the reaction (data not shown).

Os3BGlu6 E178Q and E178A showed negligible hydrolysis of *p*NPGlc within an hour, but hydrolysis products were detected after overnight reaction. When sodium azide was present, transglucosylation products were detected after overnight reaction, but no significant hydrolysis products were detected (Figure 3.29). However, when this E179Q mutant was used in an attempt to generate GA_4-GE by transglycosylation of GA_4 acceptor with *p*NPGlc donor, no transglycosylation product was detected (not shown). When GA_4-GE was used as donor and sodium azide as acceptor, both hydrolysis and transglucosylation products were detected

in the reaction of Os3BGlu6 E178Q and E178A, but transglucosylation products were apparently the main products (Figure 3.30).

Table 3.6 Hydrolysis and transglucosylation activities of Os3BGlu6 and its mutants detected by TLC

Enzyme	Donor	Acceptor	Hydrolysis	Trans-glucosylation
Os3BGlu6	*p*NPGlc	GA_4	✓	**nd**
Os3BGlu6	dNP2FGlc	GA_4	**nd**	**nd**
Os3BGlu6	*p*NPGlc	Na-azide	✓	**nd**
Os3BGlu6	GA_4-GE	Na-azide	✓	**nd**
M251N	*p*NPGlc	Na-azide	✓	**nd**
M251N	GA_4-GE	Na-azide	✓	**nd**
E178Q	*p*NPGlc	GA_4	**nd**	**nd**
E178Q	dNP2FGlc	GA_4	**nd**	**nd**
E178Q	*p*NPGlc	Na-azide	**nd**	✓
E178Q	*p*NPGlc	H_2O	✓	**nd**
E178Q	GA_4-G**E**	Na-azide	✓	✓
E178A	*p*NPGlc	Na-azide	**nd**	✓
E178A	GA_4-GE	Na-azide	✓	✓

nd: not detected. Overnight reaction products were spotted on TLC, the plates developed in appropriate solvents, and carbohydrate products were detected with sulfuric acid staining followed by charring.

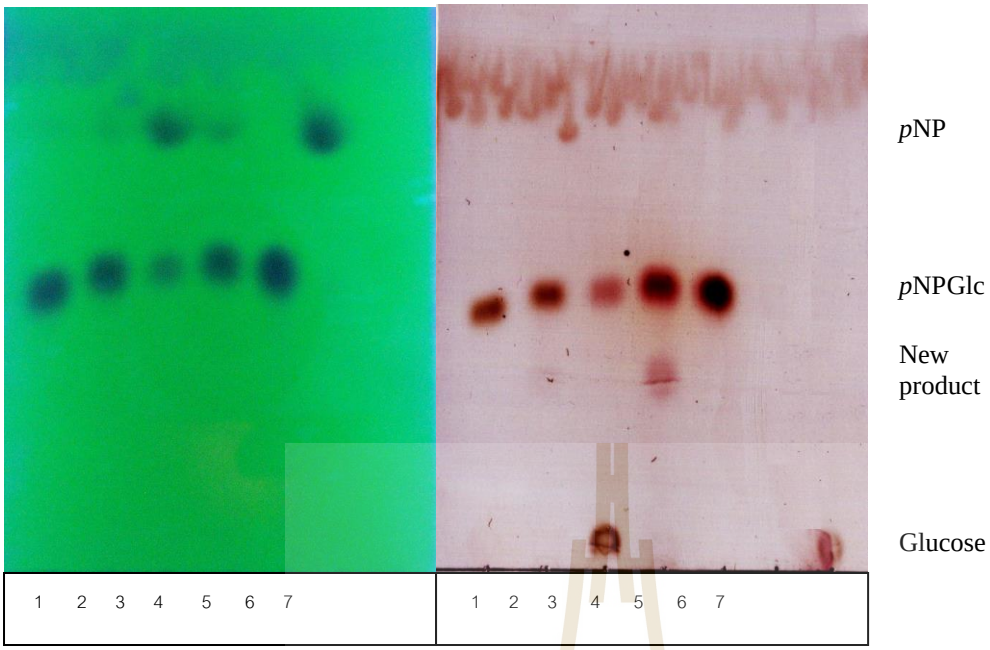

Figure 3.29 TLC analysis of the reaction of Os3BGlu6 E178Q with *p*NPGlc with and without sodium azide. In each reaction, 3.0 µg of Os3BGlu6 E178Q was reacted with 2 mM *p*NPGlc in 50 mM MES buffer at 30°C for 1 h or 24 h. The resulting reaction solutions were spotted onto a silica gel 60 F_{254} TLC plate, which was developed with EtOAc-MeOH-NH_4OH (7.0:2.8:0.2 v/v/v), and detected with UV_{254} (plate A) and then sulfuric acid staining followed by charring (plate B). Lane 1, reaction of E178Q + *p*NPGlc in MES buffer, 1 h; Lane 2, reaction of E178Q + *p*NPGlc +50 mM Na-azide in 50 mM MES buffer, 1 h; Lane 3, reaction of E178Q + *p*NPGlc in MES buffer, 24 h; Lane 4, reaction of E178Q + *p*NPGlc +50 mM Na-azide in 50 mM MES buffer, 24 h; Lane 5, *p*NPGlc standard; Lane 6, *p*NP standard; Lane 7, Glucose standard.

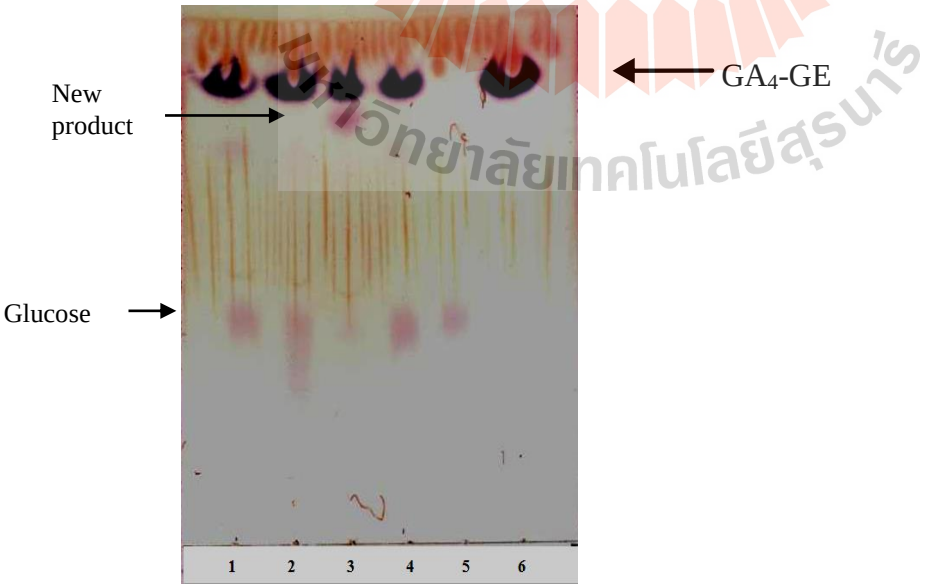

Figure 3.30 Reaction of Os3BGlu6 E178A with GA_4-GE in different solutions. In each case, 3.5 µg of Os3BGlu6 E178A was reacted with 5 mM GA_4-GE in a different buffer at 30°C for

60 min. The developing solvent was EtOAc-MeOH-H_2O (7.5:2.5:1.0 v/v/v), and carbohydrate products were detected with sulfuric acid staining followed by charring. Lane 1: reaction in 50 mM NaOAc; Lane 2: reaction in universal buffer; Lane 3: reaction with 50 mM Na-azide in 50 mM MES; Lane 4: reaction in 50 mM MES; Lane 5: Glucose standard; Lane 6: GA_4-GE standard.

The transglucosylation products of Os3BGlu6 E178A with GA_4-GE observed in TLC (Figure 3.30) was expected to be β-D-glucopyranosyl azide (also called 1-azido-β-D-glucose) based on the retaining mechanism of the GH1 β-glucosidases. This product had a mass spectrum consistent with the molecular mass of 205.17 of β-D-glucopyranosyl azide (data not shown) confirming it was likely the expected product.

The structure of the β-D-glucopyranosyl azide was also confirmed from its ^1H and gCOSY NMR spectra. The ^1H spectrum peaks were assigned as: δ4.53, H1, d, $J_{2,1}$=9.0 Hz; δ3.85, H6, dd, $J_{6,6'}$=11.1 Hz; δ3.678, H6′, dd, $J_{5,6'}$=4.8Hz, $J_{6,6'}$=11.1 Hz; δ3.364-3.458, H3,H4, H5, m; δ3.192, H2, t, $J_{1,2}$ = $J_{3,2}$ =9.0 Hz. The ^1H NMR spectrum matched the published data for β-D-glucopyranosyl azide (Wang et al., 1995). The coupling constant between H1 and H2 of 9.0 Hz, confirmed the β-D-glucopyranosyl azide had the "β" configuration.

3.5.4 Transglucosylation kinetics of the mutants of Os3BGlu6

The activity versus pH profiles for transglucosylation of azide with GA_4-GE donor were determined in NaOAc and MES buffers. For the Os3BGlu6 E178Q mutant, its optimum pH range was 5.0 to 6.0 in both buffers (Figure 3.31), slightly higher than its optimum pH for hydrolysis of GA_4-GE. The Os3BGlu6 E178A mutant showed a different pattern. Its transglucosylation activity was high at pH 5 in MES buffer, but high at pH 4.0 in NaOAc buffer. In NaOAc buffer, the transglucosylation activity of the Os3BGlu6 E178A was much higher than that in MES buffer, even without azide in the system. This indicated that NaOAc could act as a nucleophile or substitute acid/base to rescue the hydrolysis activity.

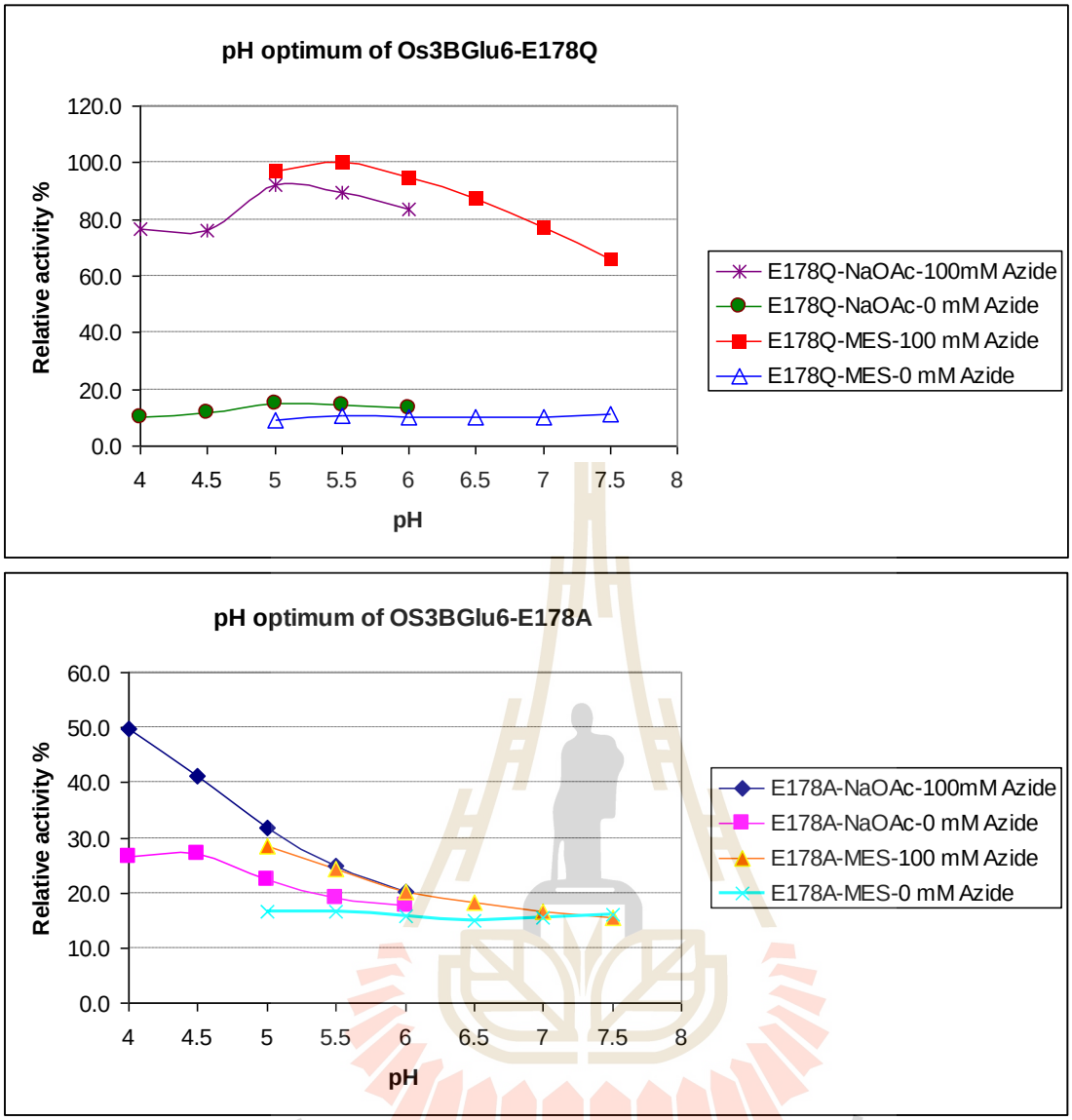

Figure 3.31 pH dependence of transglucosylation of azide with GA_4-GE donor by Os3BGlu6 E178Q (Top) and E178A (Bottom) mutants. In each reaction, 2 µg of Os3BGlu6 E178Q or E178A was incubated with 2 mM GA_4-GE and 100 mM Na-azide, in 50 mM MES (pH 5.0-7.5) and 50 mM NaOAc buffer (pH 4.0-6.0) at 30°C for 30 min. The relative activities were measured from the released GA_4. The highest activity of Os3BGlu6 E178Q with 100 mM azide in MES buffer at pH 5.5 was set as 100% for relative activities in both figures.

Figure 3.32 shows that when the concentration of donor GA_4-GE was fixed at 2 mM, the turnover rates V_0/E_0 of GA_4 for Os3BGlu6 E178A slowly increased with increasing concentrations of Na-azide and reached its maximum at 400 mM Na-azide. However, for Os3BGlu6 E178Q, the turnover rates of GA_4 slowed down after reaching a maximum at 100 mM Na-azide. This indicates that the high concentration of Na-azide was inhibiting the reaction. Two methods were tried for calculating the kinetic parameters; one was calculated from the released GA_4 after subtracting hydrolysis product and another one without subtracting hydrolysis product. Since transglucosylation products were the main products and little glucose was evident when the acceptor azide was present compared to when it was not, the parameters calculated from the released GA_4 without subtracting hydrolysis should yield a

better estimate for these reactions. The k_{cat} /K_m values of Os3BGlu6 E178 A and Os3BGlu6 E178Q were 0.066 and 0.13 $mM^{-1}s^{-1}$, respectively, when they were calculated from the released GA_4 without subtracting hydrolysis product (Table 3.7).

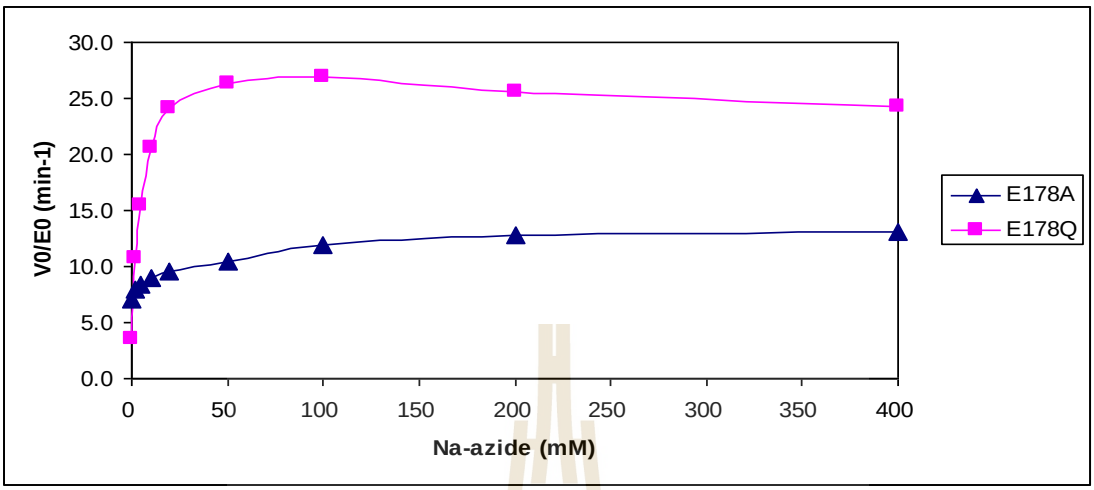

Figure 3.32 Kinetics for transglucosylation of azide acceptor with GA_4-GE donor by the Os3BGlu6 E178A and E178Q acid/base mutants. The concentrations of Na-azide acceptor were varied from 0 to 400 mM, while donor GA_4-GE was fixed at 2 mM. The reactions were performed in 50 mM MES, pH 5, at 30°C for 20 min. The turnover rates, V_0/E_0, of GA_4 (µmole of GA_4 release per minute per µmole of enzyme) were calculated based on the area of GA_4 HPLC peaks. The total area of GA_4 was used without subtracting the hydrolysis reaction.

Table 3.7 Transglucosylation kinetics of Os3BGlu6 E178Q and E178A for sodium azide acceptor with 2 mM GA_4-GE donor.

Enzyme	**Na-azide**		
	K_m (mM)	k_{cat} (s^{-1})	k_{cat} /K_m ($mM^{-1}s^{-1}$)
Os3BGlu6 E178Q (a)	5.78 ± 0.37	0.44 ± 0.01	0.075
(b)	3.71 ± 0.47	0.48 ± 0.01	0.13
Os3BGlu6 E178A (a)	30.1 ± 3.1	0.106 ± 0.003	0.0035
(b)	3.1 ± 0.5	0.204 ± 0.006	0.066

The transglucosylation rates were calculated from the released GA_4 after subtracting hydrolysis product from the reaction without azide (a) and without subtracting hydrolysis product (b).

The effects of the concentration of Na-azide on the transglucosylation kinetics were further studied by varying concentrations of donor GA_4-GE, fixing the concentration of Na-azide at 50, 100, 200 and 400 mM, and measuring k_{cat} and K_m for GA_4-GE. Figure 3.33 shows that 400 mM Na-azide did not inhibit the transglucosylation reaction of Os3BGlu6 E178A yet, but 200 mM and 400 mM Na-azide showed significant inhibition in the reactions catalyzed by Os3BGlu6 E178Q. The kinetic parameters also confirmed this inhibition (Table 3.8). For Os3BGlu6 E178Q, the k_{cat}/K_m of GA_4-GE was 0.51 when the concentration of Na-azide was 100 mM, and reduced to 0.41 and 0.29 when the concentration of Na-azide was increased to 200 and 400 mM, respectively. For Os3BGlu6 E178A, the k_{cat}/K_m of GA_4-GE was not changed when the concentration of Na-azide increased to 400 mM from 200 mM.

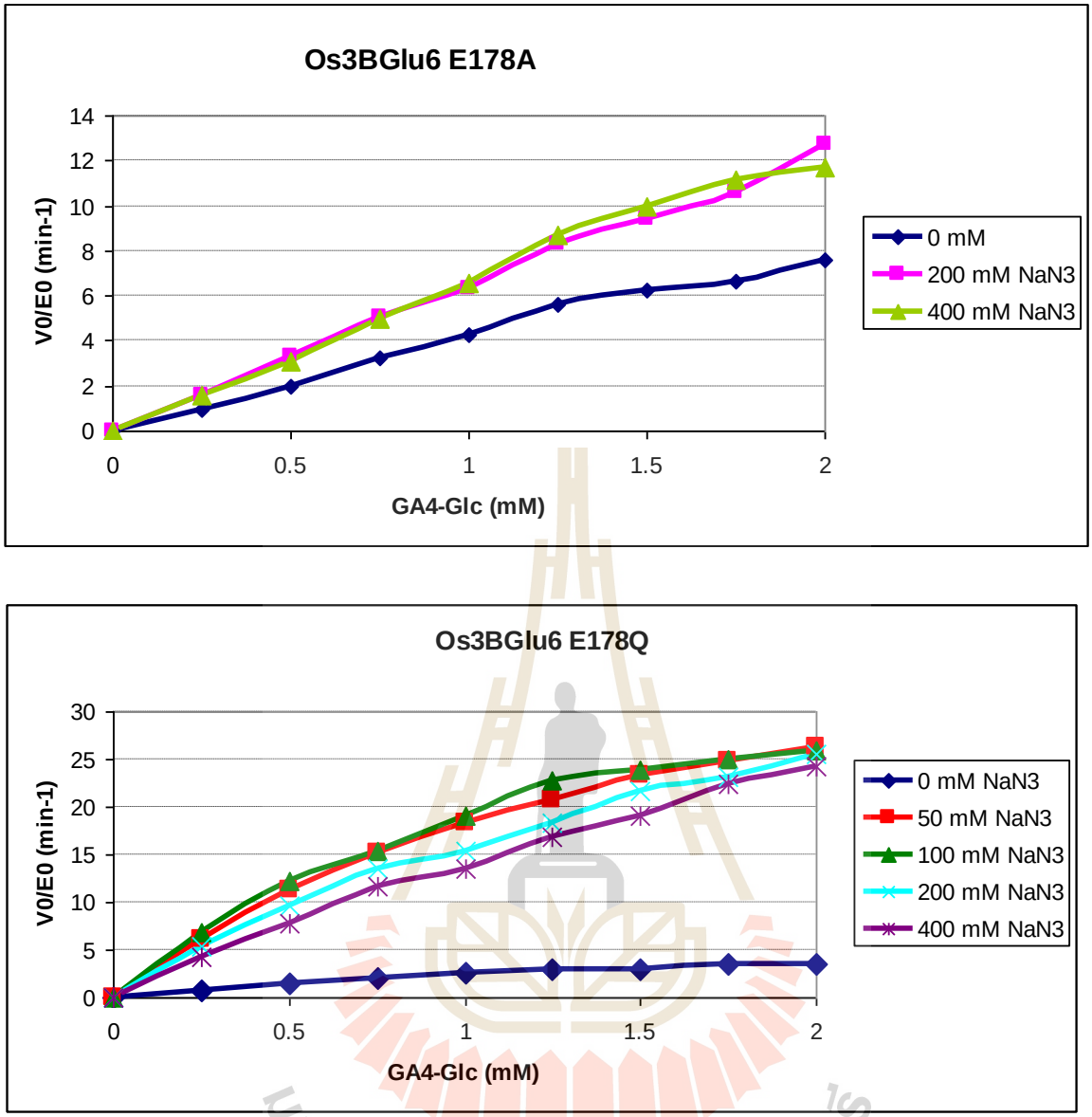

Figure 3.33 Reaction rates for transglucosylation of different concentrations of azide acceptor with GA_4-GE donor by Os3BGlu6 acid/base mutants. Top, Os3BGlu6 E178A; bottom, Os3BGlu6 E178Q. The concentrations of donor GA_4-GE were varied, while Na-azide was fixed at 50, 100, 200 and 400 mM, and reactions without Na-azide were used as controls. The reactions were performed in 50 mM MES, pH 5, at 30°C for 20 min. The turnover rates V_0/E_0 of GA_4 (µmole of GA_4 release per minute per µmole of enzyme) were calculated based on the areas of GA_4 HPLC peaks. The total area of GA_4 was used without subtracting hydrolysis product, since glucose was not readily detected when azide was present in the reaction.

Table 3.8 Transglucosylation kinetics of Os3BGlu6 E178Q and E178A for GA_4-GE donor at various fixed concentrations of sodium azide acceptor.

Enzyme	Na-azide (mM)	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($mM^{-1}s^{-1}$)
Os3BGlu6	0	0.09 ± 0.01	0.03 ± 0.001	0.33
E178Q	50	1.64 ± 0.15	0.81 ± 0.04	0.49
	100	1.69 ± 0.13	0.86 ± 0.04	0.51
	200	2.07 ± 0.31	0.84 ± 0.08	0.41
	400	3.97 ± 0.65	1.16 ± 0.14	0.29
Os3BGlu6	0	3.7 ± 0.4	0.09 ± 0.005	0.03
E178A	200	11.6	1.39	0.12
	400	13.36	1.56	0.12

The transglucosylation rates were calculated from the released GA_4 without subtracting hydrolysis product.

CHAPTER 4. Analysis

4.1 Discussion

4.1.1 Syntheses of gibberellin glucosyl conjugates

Deacetylated GA_4-GE and GA_3-GE esters were synthesized with final yields of 40% and 10%, respectively. Monitoring purification of β-glucosidase requires a lot of substrate, which must be custom synthesized for GA glucosyl conjugates. Considering the difficulty in synthesis of these conjugates and the fact that although GA_4 and its conjugates were discovered in rice, and neither GA_3 nor its conjugates were found in rice [*MacMillan*, 2002; **Sembdner et al., 1994**], GA_4-GE was chosen as the substrate to test the β-D-glucosidase activity. Therefore, we undertook purification of a β-glucosidase that can hydrolyze GA_4-GE from rice.

4.1.2 Extraction, purification and characterization of β-glucosidase from rice

From ten kilograms of 10-day rice seedling shoots and leaves, 0.15 mg of protein was obtained, for which the specific activities to *p*NPGlc and GA_4-GE were 10.9 and 0.14 μmol/min.mg, respectively. The purification of GA_4-GE-hydrolyzing β-D-glucosidase was only 337 fold, which is rather low considering the low yield of the protein. The total activity of final protein was only 0.18% of the original protein. The purification work lasted one month and many steps had to be done at room temperature (25-30°C). During the purification, some of the enzyme molecules may have been degraded or lost their activity, which may be one reason for the low purification yield. In addition, some isoenzymes that have GA_4-GE-hydrolyzing ability (many with lower specific activity than the purified protein) were likely to have been removed during the purification, resulting in a decrease in yield.

In the final protein, two major bands with size at approximately 60 kDa were observed in the 10% SDS-PAGE. They co-eluted in the SP cation exchange column at pH 5, octyl sepharose 4 hydrophobic interaction column and S200 gel filtration column. The final protein mix was submitted to two further purification attempts with SP Sephadex cation exchange chromatography in buffers at pH 5.5 and 6.0, respectively. The protein could not bind to the column at these two pH values; although previously they were bound to the SP column at pH 5.0. A Resource Q anion exchange column was also tested to see whether it could resolve the remaining bands. The column was eluted with a linear gradient of 0-1.0 M NaCl in 4-fold diluted McIlvaine buffer, pH 7 and pH 8, respectively, but the protein did not bind to the column at either pH.

The two major protein bands from the final purified protein fractions were identified by LCMS after tryptic digestion to peptides. The peptide masses from the upper band matched a hypothetical protein OsI_23311 [*Oryza sativa* Indica Group] with the matching score of 851. The protein was predicted to have a molecular mass of 57,128 Da, and calculated pI of 8.67 for the precursor, including its signal peptide. The first 28 residues were predicted as a signal sequence in the precursor protein by SignalP 4.0 (Petersen et al., 2011); and 3 N-glycosylation sites at residues 33, 168 and 326 were predicted with NetNGlyc 1.0 (Gupta et al., 2004) in the amino acid sequence of the precursor protein.

The molecular mass and theoretical pI of the mature protein were predicted to be 54,150 Da and 8.74, respectively. Since this protein bound to the Con A column, it is likely to be glycosylated, which may easily account for its slightly larger apparent size of 60 kDa on

the SDS-PAGE. Although this protein has a region homologous to an FAD-binding domain, the function of this protein is not clear yet.

The peptide masses from the lower band matched the protein product from the Os04g0474900 gene locus [*Oryza sativa* Japonica Group], which corresponds to a GH1 β-glucosidase Os4BGlu13, with the matching score of 1006 and sequence coverage of 49%. This precursor protein was calculated to have a molecular mass of 57,386 Da and pI of 6.44 when its signal peptides were included. The first 25 residues were predicted to be a signal sequence in the precursor protein by SignalP 4.0 (Petersen et al., 2011); the molecular mass and theoretical pI after the signal peptide was removed were predicted to be 54,761 Da and 6.66, respectively. Considering the fact that there are five putative N-linked glycosylation sites in the amino acid sequence of the precursor protein, this molecular mass matched the size seen in the 10% SDS-PAGE (Figure 3.40).

The β-D-glucosidase Os4BGlu13, also called OsTAGG1, was previously reported by Wakuta et al. (2010) as a β-D-glucosidase hydrolyzing tuberonic acid glucoside (TAG). The protein was purified from rice panicles by $(NH_4)_2SO_4$ fractionation and five different types of chromatography. The purified OsTAGG1 migrated as a single band on native PAGE, but was present as two polypeptides with molecular masses of 42 kDa and 26 kDa under denaturing conditions. They explained that the two apparent subunits were caused by proteolytic cleavage of the initial mature protein to produce two bands in SDS-PAGE, and they determined the two N-terminal sequences. The sum of the molecular masses of two polypeptides was 68 kDa, which is different from the predicted molecular mass of 55 kDa. This discrepancy was explained by authors as likely to be due to the fact that there are five N-linked glycosylation sites in the amino acid sequence of the precursor protein, so the molecular size of the mature protein could be increased by addition of sugar chains.

In conclusion, Os4BGlu13 β-D-glucosidase was purified from rice seedling shoots and leaves, but with only approximate 50% purity. This protein could hydrolyze GA_4-GE to release GA_4, and also hydrolyze TAG to release TA with high activity. Wakuta et al. (2010) purified the same protein, but with different molecular size, as judged by SDS-PAGE. From our purification, we observed a single protein band on SDS-PAGE, but Wakuta et al. reported two bands on SDS-PAGE. Our enzyme was extracted from 10-day old rice seeding shoots and leaves, while Wakuta's enzyme was extracted from rice panicles during grain filling. So, the different tissue sources may be the main cause for obtaining two different molecular sizes for the same mature protein, if the protein undergoes proteolytic processing in the panicle that does not occur in the seedling shoots and leaves, and different levels of glycosylation occur in the different tissues. Since different purification methods were used, Wakuta's protein may have been proteolytically cleaved during the purification, while this proteolysis did not occur in our work.

4.1.3 Hydrolysis of GA_4-GE by Os3BGlu6 and its mutants

Five rice GH1 enzymes that have been expressed in our lab, Os3BGlu6, Os3BGlu7, Os4BGlu12, Os3BGlu18 and Os9BGlu31, were tested for the hydrolysis of *p*NPGlc and GA_4-GE (Table 3.4). Os3BGlu6 was found to have the highest hydrolysis activity to GA_4-GE among these enzymes. Although Os4BGlu12 could hydrolyze *p*NPGlc with high activity, its hydrolysis activity toward GA_4-GE was only 20% that of Os3BGlu6. Since Os4BGlu12 is the isoenzyme most closely related to Os4BGlu13, which was identified as the GA_4-GE β-glucosidase purified from the plant, this was a little unexpected. Therefore, Os3BGlu6 hydrolyzes GA_4-GE better and with a higher preference than Os4BGlu12 and other rice GH1

β-D-glucosidases, such as Os3BGlu7. We have not expressed Os4BGlu13 in the same system, so it is difficult to compare Os3BGlu6 and Os4BGlu13.

Although much characterization has been done for the hydrolysis kinetics of alkyl and aryl glycosides and oligosaccharides by β-D-glucosidases and their catalytic acid/base and nucleophile mutants (Wang et al., 1994, 1995; Mackenzie et al., 1998; Chuenchor et al., 2011), little or no description of glucosyl ester hydrolysis is available in the literature. Since Os3BGlu6 was identified as a β-D-glucosidase with relatively high ability to hydrolyze GA_4-GE ester, it serves as a good model for investigation of β-glucosidic hydrolysis of glucosyl esters. It might be expected that the relatively low pK_a of the leaving group would make the glucosyl ester relatively insensitive to the presence of a catalytic acid for the initial glycosylation step, similar to 2,4-dinitrophenyl glycosides, but other influences of having the ester bond in apposition to the glycosidic bond are not so clear. In order to study the properties of glucosyl ester hydrolysis and glucosyl transfer by a β-glucosidase, the wild type rice Os3BGlu6 and its catalytic acid/base mutants E178Q and E178A were expressed and purified, and their optimum pH and activities for hydrolysis of GA_4-GE and *p*NPGlc were compared. The Os3BGlu6 active site cleft mutant M251N, which was previously created in our laboratory to improve hydrolysis of cellooligosaccharides by Os3BGlu6 [Sansenya et al., 2012], was also tested. The activity of two catalytic nucleophile mutants developed for possible production of Os3BGlu6 GA_4-GE ester complexes for structural analysis, E394D and E394Q, were also tested, since loss of the nucleophile is expected to greatly decrease the enzyme activity regardless of the substrate, as described by Wang et al. [1994].

The Os3BGlu6 wild type and M251N mutant were found to have high *p*NPGlc hydrolysis activity between pH 4.0 and 5.0; and Os3BGlu6 wild type and its M251N, E178Q and E178A mutants also showed highest GA_4-GE hydrolytic activities at pH 4.5. This optimum pH is in the range of commonly seen in plant β-glucosidases [Esen, 1993] and agreed with the previously reported pH optimum for wild type Os3BGlu6 [Seshadri et al., 2009]. When hydrolyzing *p*NPGlc, the activity of Os3BGlu6 wild type and M251N quickly dropped above pH 5.5 (Figure 3.29). In contrast, the Os3BGlu6 E178Q, E178A, E394D and E394Q mutants had less than 1% of wildtype activity toward *p*NPGlc. Although the catalytic nucleophile mutants had little activity to GA_4-GE as well, the activities of Os3BGlu6 E178Q and E178A were significant and dropped slowly above pH 6.0 when hydrolyzing GA_4-GE, compared to wild type Os3BGlu6 and Os3BGlu6 M251N. The pH dependence of enzymatic reactions is generally considered to reflect ionizations of acid/base groups involved in catalysis (Kempton and Withers, 1992; McIntoch et al., 1996). The residue E178 in Os3BGlu6 acts as a general acid catalyst to protonate the glycosidic oxygen during the enzyme glycosylation step, and then as a general base to deprotonate the attacking water during deglycosylation. The dual role of E178 places specific demands upon its ionization states, which has been seen for E172 in *Bacillus circulans* xylanase in McIntoch's work (1996). For Os3BGlu6 E178Q and E178A, the glutamate residue was mutated to glutamine and alanine, so the pH had no effect on ionization of the sidechain and its effect on the hydrolysis was reduced. This is why the activities of Os3BGlu6 E178Q and E178A were not very sensitive to the pH compared to Os3BGlu6 wild type and M251N when hydrolyzing GA_4-GE.

The structures of the Os3BGlu6 apo enzyme, its complexes with 2-deoxy-2-fluoro glucoside and n-octyl-β-D-thioglucoside have been solved by X-ray crystallography (Seshadri et al., 2009). In the active site, E178 and E394 were positioned as the two catalytic residues. M251 was located at the mouth of active site cleft and appeared to block the binding of extended β-(1/4)-linked oligosaccharides and interact with the hydrophobic aglycone of n-

octyl-β-D-thioglucopyranoside. A few nonpolar aromatic residues W366, W133 and F460 were located around the cleft. This correlates with the preference of Os3BGlu6 for short oligosaccharides and hydrophobic glycosides. Mutation of Os3BGlu6 Met251 to Asn resulted in a 15-fold increased k_{cat}/K_m value for hydrolysis of laminaribiose compared to wild type Os3BGlu6 and 9 to 24-fold increases for cellooligo-saccharides with degrees of polymerization (DP) of 2-5 (Sansenya et al., 2012). These results indicated that conversion of Met to Asn could generate an oligosaccharide binding subsite in Os3BGlu6, and increase its hydrolytic activity for oligosaccharide. In our study, the Os3BGlu6 M251N mutant showed reduced hydrolytic activities for *p*NPGlc and GA_4-GE compared to its wild type. The relative activities of M251N compared to wildtype Os3BGlu6 were 52% for *p*NPGlc and 89% for GA_4-GE, while the catalytic efficiencies (k_{cat}/K_m) for M251N were reduced to 2.6 from 6.2 $mM^{-1}s^{-1}$ for *p*NPGlc, and 0.08 from 0.13 for GA_4-GE compared to wild type. The K_m to *p*NPGlc for both Os3BGlu6 and Os3BGlu6 M251N is almost the same; the smaller k_{cat} of Os3BGlu6 M251N is the main cause for its lower k_{cat}/K_m compared to the wild type. This suggested that the two enzymes' affinities for *p*NPGlc are similar in the binding substrate step due to the smaller size of *p*NPGlc, which would not reach to the M251 residue from a position with it glycosidic bond in position for hydrolysis, but Os3BGlu6 M251N has lower k_{cat} than Os3BGlu6. In contrast, for GA_4-GE, the higher K_m for Os3BGlu6 M251N is the main reason for its lower k_{cat}/K_m compared to the wild type. This suggested that N251 residue in Os3BGlu6 M251N reduced the affinity to GA_4-GE due to the bulky size of the GA_4-GE and the polarity of N251 residue. The higher K_m of Os3BGlu6 M251N led to its lower k_{cat}/K_m. This suggests that Met251 functions to stabilize hydrophobic aglycones of these substrates in the reaction processing.

The generally accepted catalytic mechanism of family 1 glycoside hydrolase was confirmed for Os3BGlu6 in this work. As with other GH Clan A families, GH1 enzymes act through a retaining mechanism, utilizing one conserved glutamate residue as a catalytic acid/base and a second as a nucleophile or base [Rye and Withers, 2000]. The two-step mechanism with a covalent enzyme-β-glucoside intermediate (Figure 4.1) has been supported by the demonstration of covalent intermediates of 2-F-glucosides [Withers et al., 1990] and natural glucoside with an acid/base mutant [Noguchi et al., 2008]. In the glycosylation step, the catalytic nucleophile of the enzyme displaces the aglycone with general acid-assistance from the catalytic acid/base to form the intermediate. The deglycosylation step is the reverse of the first step, with water or another nucleophile attacking from the opposite side, with basic assistance from the catalytic acid/base, to displace the enzyme from the glycone. If the displacing nucleophile is water, hydrolysis results; whereas with other nucleophiles, transglycosylation results. In our study, the mutation of the glutamate at residue 178 to alanine (E178A) and glutamine (E178Q) led to complete loss of hydrolytic activity toward *p*NPGlc; and the relative activities to GA_4-Glc were reduced to 22.2% and 12.5%, respectively, compared to its wild type. The mutation of glutamate residue 394 to glutamine (E394Q) and aspartic acid (E394D) led to complete loss of hydrolytic activity toward *p*NPGlc and GA_4-GE. These data support the identification of E178 as the acid/base catalyst and E394 as a nucleophile in the process. Since the pK_a of GA_4 and *p*NP are 4.3 and 7.2, respectively, the -COO attached on GA_4 is a better leaving group than *p*-nitrophenolate (*p*NP). Without acid-assistance from residue E178, *p*NP could not be released, so Os3BGlu6 E178A and Os3BGlu6 E78Q could not hydrolyze *p*NPGlc. Without residue E178, the GA_4 will be released less in the glycosylation step without general acid-assistance, and the water molecule cannot be deprotonated in the deglycosylation step, so Os3BGlu6 E178A and Os3BGlu6 E78Q kept partial activity to GA_4-GE compared to their wild type.

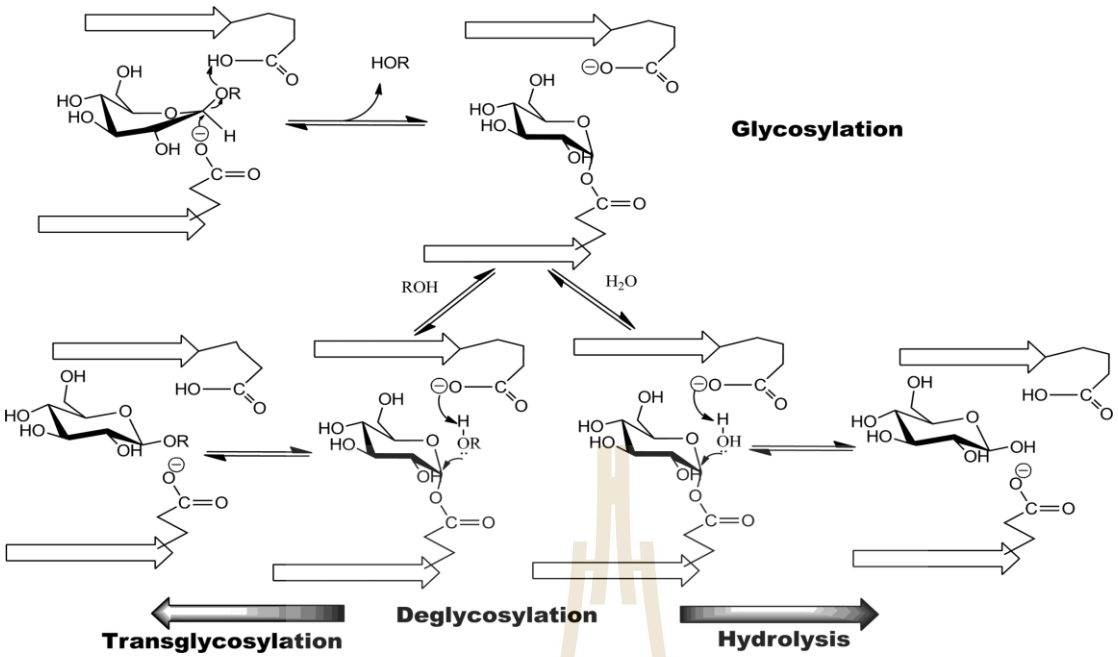

Figure 4.1 Double displacement mechanism proposed for retaining β-glycosidases, such as those in glycoside hydrolase family 1. The first step of glycosylation is shown in the top row, which forms a semi-stable glycosyl enzyme intermediate. The deglycosylation step in the bottom row is split into hydrolysis to the right and transglycosylation to the left, depending on whether water or another nucleophile acts as the acceptor substrate (from Ketudat Cairns et al., 2012).

The transglucosylation activities were discovered for Os3BGlu6 E178Q and Os3BGlu6 E178A. Wild type Os3BGlu6 and Os3BGlu6 M251N hydrolyzed *p*NPGlc in MES buffer to *p*NP and glucose only, and no transglucosylation products were formed when GA_4 and azide were added as acceptors. For Os3BGlu6 E178Q and E178A, when *p*NPGlc or GA_4-GE was used as donor and sodium azide as acceptor, both could produce transglucosylation products. Without sodium azide, only hydrolysis products were observed, while with sodium azide, the transglucosylation product β-glucosyl azide was the main product. This indicates that hydrolysis and transglucosylation are two competing reactions and that the strong nucleophile sodium azide is better at displacing the enzyme from the glycone in the absence of a catalytic base.

The transglucosylation kinetics were also studied for the acid/base mutants of Os3BGlu6. The activity versus pH profiles for transglucosylation of azide with GA_4-GE donor were determined in NaOAc and MES buffers. For the Os3BGlu6 E178Q mutant, its optimum pH range was 5.0 to 6.0 in both buffers, slightly higher than its optimum pH for hydrolysis of GA_4-GE. The E178A mutant showed a different pattern. Its transgluco-sylation activity was high at pH 5 in the MES buffer, but high at pH 4.0 in the NaOAc buffer. In the NaOAc buffer, the GA_4 releasing activity of the E178A was much higher than the one in MES buffer, even without azide in the system. This indicated that acetate could act as a nucleophile or substitute acid/base to rescue the hydrolysis activity. The work of Wang et al. (1995) also showed that the rate of hydrolysis of 2,4-dinitrophenyl β-D-glucopyranoside (2,4-DNPG) by the *Agrobacterium* β-glucosidase acid/base mutant AbgE178G could be increased by nucleophiles, such as azide, acetate, formate, benzoate, and thiophenyl to 292, 159, 150,

67 and 15 fold, respectively, compared to the reaction without nucleophile present. This is also one reason why we selected MES buffer for the transglucosylation kinetic studies instead of acetate and McIlvaine buffer, to avoid rescue of the cleavage activity by nucleophiles from the buffer.

When the transglucosylation activities of Os3BGlu6 E178Q and Os3BGlu6 E178A were compared with GA_4-GE donor fixed at 2 mM and varying concentrations of sodium azide as acceptor, the k_{cat}/K_m values for Os3BGlu6 E178Q and Os3BGlu6 E178A were 0.13 and 0.066 $mM^{-1}s^{-1}$, respectively. These indicate that the polar glutamine at residue 178 in Os3BGlu6 E178Q supported transglycosylation by azide better than the nonpolar alanine residue at the same position in Os3BGlu6 E178A. Since the k_{cat}/K_m value is generally thought to reflect the first covalent step, the energy of glycosyl enzyme intermediate formation at the glycosylation step (Figure 4.1) is lower for Os3BGlu6 E178Q than Os3BGlu6 E178A, likely due to hydrogen bonding to the leaving group.

Another phenomenon is that the transglucosylation turnover rate, V_0/E_0, of GA_4–GE was increased very significantly as the concentration of azide was increased. The maximum of V_0/E_0 for Os3BGlu6 E178Q was seen at 100 mM of azide, and this specific activity gradually decreased when the concentration of azide increased further. In contrast, for Os3BGlu6 E178A, this inhibition was observed when the concentration of azide reached 400 mM. The rate increase with increasing concentration of azide was also observed by Wang et al. (1995). They concluded that addition of azide as a competitive nucleophile increased k_{cat} values 100-300 fold for substrates whose rate-limiting step is deglycosylation, but had no effect on the wild type enzyme. For the hydrolysis of GA_4-GE with Os3BGlu6 E178Q and Os3BGlu6 E178A, the rate-limiting step is deglycosy-lation. As the concentration of azide is increased, the rate of deglycosylation increases, until glycosylation becomes rate determining. The inhibition of transglucosylation of Os3BGlu6 E178Q by high concentrations of azide might be due to the high concentrations of azide interfering with the nucleophile E394 in the process of attacking the anomeric carbon during glycosylation, thus reducing the reaction rate. Actually, high concentrations of azide were also observed to inhibit hydrolysis of GA_4-GE by wild type Os3BGlu6.

4.1.4 Comparison of the protein structures and substrate binding for Os3BGlu6, Os3BGlu7, Os4BGlu12 and Os4BGlu13

To study the relationship between the protein's structure and their substrate binding, the amino acid sequences of Os3BGlu6, Os3BGlu7 and Os4BGlu12 were aligned with Os4BGlu13. The alignment results (Figure 4.2) show that Os4BGlu12 and Os4BGlu13 have 85% identity; Os3BGlu6 and Os3BGlu7 have 49% identity with Os4BGlu13. The identity between Os3BGlu6 and Os3BGlu7 is also 49%.

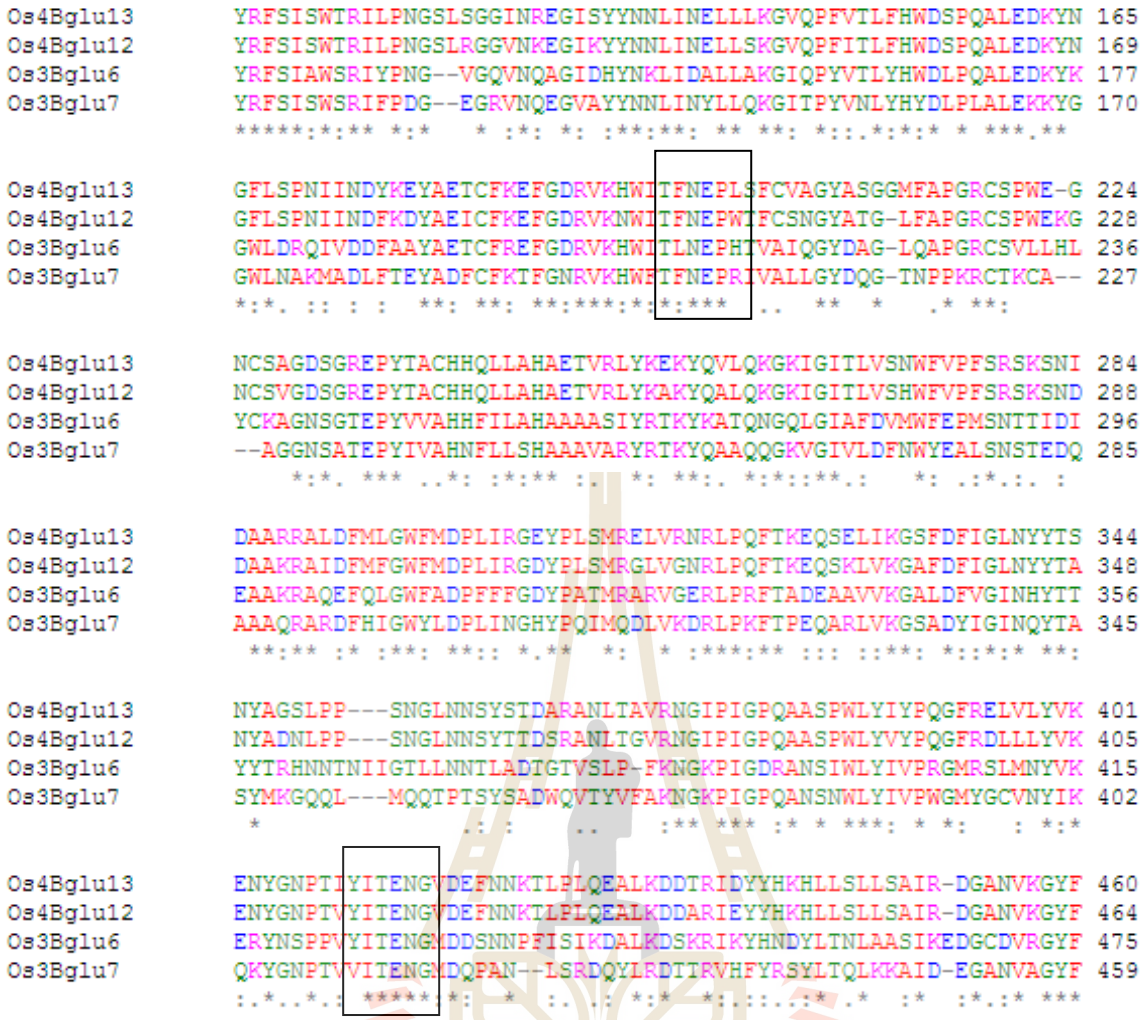

Figure 4.2 Sequence alignment for four rice β-glycosidases, Os3BGlu6, Os3BGlu7, Os4BGlu12 and Os4BGlu13. Only portions of each polypeptide are shown. The regions surrounding the two catalytic amino acids are boxed in rectangles.

The sequence alignment of four β-glycosidases showed that there are two conserved amino acid residue regions near the catalytic amino acids, one is T(F/L)NEP, another one is ITENG (Figure 4.2). The glutamate residues at positions 199 and 413 (Os4BGlu13 numbering) were predicted to be the acid-base catalyst and catalytic nucleophile, respectively, based on previous studies on Os3BGlu6 (Seshadri et al., 2009), Os3BGlu7 (Opassiri et al., 2003) and Os4BGlu12 (Sansenya et al., 2011). Though Os4BGlu12 and Os4BGlu13 have 85% amino acid sequence identity, these two enzymes have shown different specific activity, especially to GA_4-GE ester. The ratio of hydrolysis activities to GA_4-GE and *p*NPGlc for partially purified Os4BGlu13 (approximately 50% purity from SDS-PAGE) was 0.01, for Os4BGlu12 was 0.0003, for Os3BGlu6 was 0.07, and for Os3BGlu7 (BGlu1) was 0.005, respectively. These indicated that for the hydrolysis of GA_4-GE, Os4BGlu13 and Os3BGlu6 are much better than Os4BGlu12 and Os3BGlu7. In contrast, for the hydrolysis of *p*NPGlc, Os4BGlu12 is the best enzyme among the four enzymes.

By comparing the active sites of Os3BGlu6, Os4BGlu12, and Os4BGlu13 (Figure 4.3), we can see that the residues at the -1 site are well conserved for the three enzymes, but

the residues at the +1 and +2 sites are different. For Os4BGlu12, two residues at the +2 site, W181 and H252, are bulkier and occupy more space than the residues at the same positions in Os3BGlu6 and Os4BGlu13, so they may lead to a smaller binding cleft. This may have resulted in poor binding of GA_4-GE for Os4BGlu12, so that it was hydrolyzed poorly. Since *p*NPGlc is relatively small in size and the interactions between the aromatic residues in the active site and *p*NP ring favored the binding, the binding to *p*NPGlc for Os4BGlu12 may be the best among the three enzymes, resulting in its high ratio of hydrolysis of *p*NPGlc comparing to GA_4-GE. In contrast, the binding clefts within Os4BGlu13 and Os3BGlu6 are likely bigger than Os4BGlu12 which may make it is easier for GA_4-GE to bind at the binding cleft, so Os4BGlu13 and Os3BGlu6 were found to have higher hydrolysis activity toward GA_4-GE relative to *p*NPGlc, compared to the other enzymes.

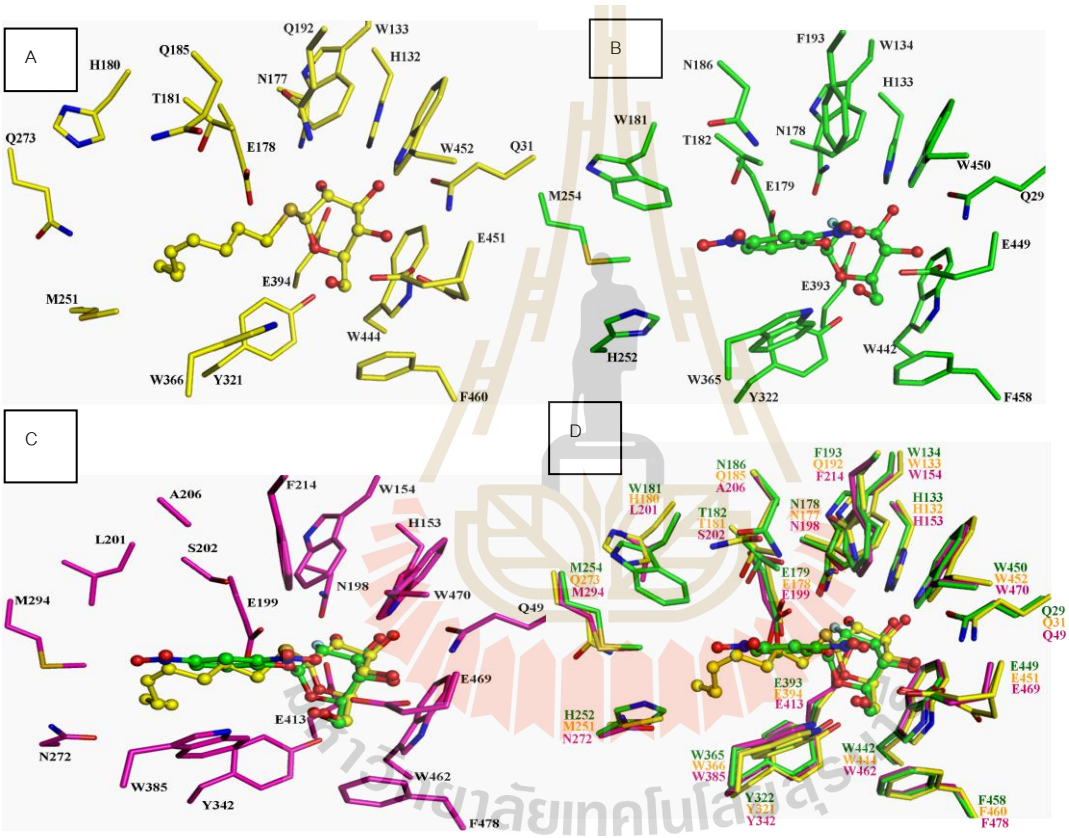

Figure 4.3 Comparison of GH1 β-glucosidase active sites.
A) The active site of the Os3BGlu6/n-octyl-β-D-thioglucopyranoside complex structure (PDB code 3GNP_A). **(B)** The active site of Os4BGlu12 with DNP2FG (PDB code 3PTQ_B). **(C)** The superimposition of the active site of a homology model of Os4BGlu13 with DNP2FG from the Os4BGlu12 complex structure (PDB code 3PTQ_B) & n-octyl-β-D-thioglucopyranoside from the Os3BGlu6 complex structure (PDB code 3GNP_A). **(D)** The superimposition of the active sites of the Os3BGlu6 complex with n-octyl-β-D-thioglucopyranoside, the Os4BGlu12 complex with DNP2FG and the Os4BGlu13 homology model. The residues with green carbons are from Os4BGlu12, those with yellow carbons are from Os3BGlu6, and those with pink carbons are from Os4BGlu13. The figures were generated by Pymol (Schrödinger LLC).

This section suggested that the amino acid residues around binding cleft are very important for the substrate binding; the charge, the polarity and the sizes of the amino acid residues all contribute to the enzyme's substrate specificity.

4.2. Conclusions and Comments

Four GA conjugates, which included acetylated and deacetylated GA_4-GE esters, acetylated and deacetylated GA_3-GE esters, were synthesized following the published methods or with modification. Their structures were identified with ^1H and gCOSY NMR and LC-MS spectrometry. The β-configuration of the H1 on the glucosyl ring were confirmed with the coupling constant of H1 ($J_{2,1}$ = approx. 8 Hz) for all glucosyl conjugates.

Ten kilograms of 10-day rice seedling shoots and leaves were extracted and the crude proteins were purified with seven purification steps based on β-D-glucosidase activities on *p*NPGlc and GA_4-GE substrates. Finally, 0.15 mg of protein was obtained, and this protein contained two major bands seen on 10% SDS-PAGE. The LC-MS analysis of these two bands after trypsin digestion indicated one protein was Os4BGlu13 β-glucosidase, the product of the Os04g074900 gene locus, which belongs to glycoside hydrolase family 1, while the other one was OsI_23311, a putative FAD binding protein.

Five rice GH1 enzymes that have been expressed in our lab, Os3BGlu6, Os3BGlu7 (BGlu1), Os4BGlu12, Os3BGlu18 and Os9BGlu31 were tested for the hydrolysis of *p*NPGlc and GA_4-GE. Os3BGlu6 was found to have the highest hydrolysis activity to GA_4-GE among these enzymes. The activity of Os4BGlu12 to hydrolyze *p*NPGlc was 50 times higher than Os3BGlu6, but the activity to hydrolyze GA_4-GE of Os4BGlu12 was only 20% of that of Os3BGlu6.

The wild type rice Os3BGlu6 and its mutants M251N, E178A and E178Q were expressed in *Escherichia coli* strain Origami (DE3) as fusion proteins with N-terminal thioredoxin and His_6 tags, and purified with 2 steps of IMAC. The proteins after the 2nd IMAC were seen as single bands and their purities were higher than 90%. The purified proteins were characterized by determination of their pH optima for hydrolysis of *p*NPGlc and GA_4-GE. The Os3BGlu6 wild type and M251N mutant were found to have high *p*NPGlc hydrolysis activity between pH 4.0 and 5.0, with highest value at pH 4.5, while the activity quickly dropped above pH 5.5. The Os3BGlu6 E178A and E178Q mutants showed little or no hydrolysis of *p*NPGlc. With GA_4-GE substrate, Os3BGlu6 wild type and M251N, E178Q and E178A mutants also showed highest hydrolytic activities at pH 4.5. The activities of Os3BGlu6 E178Q and E178A dropped slowly above pH 6.0 compared to Os3BGlu6 wild type and M251N mutant, when hydrolyzing GA_4-GE.

The activities of Os3BGlu6 wild type and its mutants to hydrolyze *p*NPGlc and GA_4-GE were analyzed. The Os3BGlu6 M251N mutant showed reduced hydrolytic activities for *p*NPGlc and GA_4-GE compared to its wild type. The relative activities of Os3BGlu6 M251N were 51.6% for *p*NPGlc, 88.9% for GA_4-GE, while the catalytic efficiencies (k_{cat} /K_m) for M251N were reduced to 2.6 from 6.2 $mM^{-1}s^{-1}$ for *p*NPGlc and to 0.08 from 0.13 for GA_4-Glc compared to wild type Os3BGlu6. The mutation of the glutamate at residue 178 to alanine (E178A) and glutamine (E178Q) led to complete loss of hydrolytic activity toward *p*NPGlc; and the relative activities to GA_4-Glc were reduced to 22.2% and 12.5%, respectively, compared to its wild type. The mutation of the E394 to glutamine (E394Q) and aspartic acid (E394D) led to complete loss of hydrolytic activity toward *p*NPGlc and GA_4-GE. This work confirmed the roles of Os3BGlu6 E178 as an acid/base catalyst and E394 as a nucleophile in the retaining catalytic mechanism of family 1 glycoside hydrolases.

The transglucosylation activities were studied for Os3BGlu6 and its mutants. Os3BGlu6 hydrolyzed *p*NPGlc in MES buffer to *p*NP and glucose, and no transglucosylation products were formed when GA_4 and azide were added as acceptors. Neither Os3BGlu6 wild type nor E178Q could hydrolyze DNP2FG, and no transglucosy-lation product was formed when GA_4 was used as an acceptor to try to rescue the reaction. When *p*NPGlc or GA_4-GE was used as donor and sodium azide as acceptor, both Os3BGlu6 E178Q and E178A could produce transglucosylation products. The prominent transglucosylation product was identified by NMR and mass spectra as β-D-gluco-pyranosyl azide.

The kinetics of Os3BGlu6 for transglucosylation of azide with GA_4-Glc donor were determined in NaOAc and MES buffers. The Os3BGlu6 E178Q and E178A mutants showed similar optimum pH ranges in MES buffer, but different pH optima in NaOAc buffer, since the acetate ion was acting as a nucleophile in the reaction to rescue the hydrolysis activity of Os3BGlu6 E178A. When the concentration of Na-azide acceptor was varied from 0 to 400 mM, while donor GA_4-GE was fixed at 2 mM, the turnover rates, V_0/E_0, of GA_4 for Os3BGlu6 E178A slowly increased with the increasing concentration of Na-azide and reached its maximum at 400 mM Na-azide. However, for Os3BGlu6 E178Q, the turnover rates of GA_4 slowed down after reaching its maximum at 100 mM Na-azide, indicating that the high concentration of Na-azide was inhibiting the reaction. For Os3BGlu6 E178Q, the k_{cat}/K_m of GA_4-GE was 0.51 when the concentration of Na-azide was 100 mM, and reduced to 0.41 and 0.29 when the concentration of Na-azide was increased to 200 and 400 mM, respectively. For Os3BGlu6 E178A, the k_{cat}/K_m of GA_4-GE was not changed when the concentration of Na-azide changed from 400 to 200 mM.

In summary, this work discovered Os3BGlu6 and Os4BGlu13 are β-glucosidases with the relatively high activity to hydrolyze GA_4-GE in the extract of rice seedlings. The hydrolysis and transglucosylation activities for Os3BGlu6 and its mutants confirmed its retaining catalysis mechanism and the roles of the residues M251, E178 and E394 in the catalytic process.

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   Biochemistry, bioinformatics, structural biology
7. ประสบการณ์ที่เกี่ยวข้องกับการบริหารงานวิจัยทั้งภายในและภายนอกประเทศ : ระบุสถานภาพในการทำการวิจัยว่าเป็นผู้อำนวยการแผนงานวิจัย หัวหน้าโครงการวิจัย หรือผู้ร่วมวิจัยในแต่ละข้อเสนอโครงการวิจัย เป็นต้น

   7.1. Journal Publications 64 publications (61 in Scopus), H-index (Scopus): 18

   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 BRG5380017.

7.2.10. 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. Head of project.

7.2.11. Characterization of a glycoside hydrolase family 1 group 6 hydrolase, 2554. SUT/NRCT, 2556/09.

7.2.12. Structure, function and application of plant β-glucosidases and related enzymes, 2556-2559, Thailand Research Fund Basic Research Grant BRG5380012. Head of project.

7.3. Projects in progress (งานวิจัยที่กำลังทำ: ชื่อแผนงานวิจัย และ/หรือโครงการวิจัย แหล่งทุน และสถานภาพในการทำวิจัยว่าได้ทำการวิจัยลุล่วงแล้วประมาณร้อยละเท่าใด)

7.3.1. Structure, Function and Application of β-Glycosidases, BRG5980017; 2559-2562, The Thailand Research Fund.

7.3.2. Characterization of rice glucosyl transferases with potential for phytohormone regulation and glycoside synthesis. 2555-2558, SUT, Budget Bureau, NRCT.

7.3.3. Characterization of Rice Phytohormone β-Glucoses, 2558-2560, SUT, Budget Bureau, NRCT.

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7.1 Journal Publications

7.1.1 Charoenwattanasatien R, Pengthaisong S, Breen I, Mutoh R, Sansenya S, Hua Y, Tankrathok A, Wu L, Songsiriritthigul C, Tanaka H, Williams S.J, Davies G.J, Kurisu G, Ketudat Cairns JR (2016): Bacterial β-Glucosidase Reveals the Structural and Functional Basis of Genetic Defects in Human Glucocerebrosidase 2 (GBA2). ACS Chem. Biol. 11 (7): 1891–1900.

7.1.2 Rimlumduan T, Hua Y, Tanaka T, Ketudat Cairns JR (2016). Structure of a plant β-galactosidase C-terminal domain. Biochimica et Biophysica Acta (BBA) - Proteins & Proteomics. 1864: 1411–1418.

7.1.3 Komvongsa J, Mahong B, Phasai K, Hua Y, Jeon JS, Ketudat Cairns JR (2015): Identification of Fatty Acid Glucose Esters as Os9BGlu31 Transglucosidase Substrates in Rice Flag Leaves. Journal of Agricultural and Food Chemistry. 63: 9764-9769.

7.1.4 Hua Y, Ekkhara W, Sansenya S, Srisomsap C, Roytrakul S, Saburi W, Takeda R, Matsuura H, Mori H, Ketudat Cairns JR (2015). Identification of rice Os4BGlu13 as a β-glucosidase which hydrolyzes gibberellin A4 1-O-β-d-glucosyl ester, in addition to tuberonic acid glucoside and salicylic acid derivative glucosides. Archives of Biochemistry and Biophysics. 583: 36-46.

7.1.5 Baiya S, Hua Y, Ekkhara W, Ketudat Cairns JR (2014). Expression and enzymatic properties of rice (Oryza sativa L.) monolignol β-glucosidases. Plant Science. 227: 101-109.

7.1.6 Boonanuntanasarn, S., Khaomek, P., Pitaksong, T., and Hua, Y (2014). The effects of the supplementation of activated charcoal on the growth, health status and fillet composition-odor of Nile tilapia (Oreochromis niloticus) before harvesting. Aquacult Int. 22: 1417–1436.

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7.1.8 Luang S, Cho J-I, Mahong B, Opassiri R, Akiyama T, Phasai K, Komvongsa J, Sasaki N, Hua Y, Matsuba Y, Ozeki Y, Jeon J-S, Ketudat Cairns JR (2013). Os9BGlu31 is a transglucosidase with the capacity to equilibrate phenolpropenoid, flavonoid and phytohormone glycoconjugates. Journal of Biological Chemistry 288(14): 10111-10123.

7.1.9 Natteewan, U, Sureelak, R, Yeung Joon C, Hua, Y, and Jirawat, Y (2011). Use of tetragenococcus halophilus as a starter culture for flavor improvement in fish sauce fermentation. J. Agri. and Food Chem. 59(15): 8401-8408.

7.1.10 Chuankhayan P, Hua Y, Svasti J, Sakdarat S, Sullivan PA, and Ketudat Cairns JR. (2005). Purification of an isoflavonoid 7-O-β-apiosyl-glucoside β-glycosidase and its substrates from Dalbergia nigrescens Kurz. Phytochemistry 66: 1880-1889.

7.1.11 Opassiri R, Hua Y, Wara-Aswapati O, Akiyama T, Svasti J, Esen A, and Ketudat Cairns JR. 2004. β-Glucosidase, exo-β-glucanase and pyridoxine transglucosylase activities of rice BGlu1. Biochem. J. 379: 125-131.

7.1.12 Flood, A.E., Pantaraks P, W. Monkaew, Y.L. Hua, A Study of the Mutarotation Reaction in Solutions of Glucose and Fructose. Proceedings of the Regional Symposium on Chemical Engineering 1999, November 22-24, Songkhla, Thailand.

7.1.13 S.S. Chen, Y.L. Hua, Z.P. Lei, X.K. Yao (1991). The Reaction of 6,6-Dialkylfulvenes with Thienyllithium -- Synthesis and Molecular Structure of Thienyl-Cyclopentadienyl Titanium and Zirconium Derivatives, Progress in Natural Science 1: 544.

7.1.14 S.S. Chen, Y.L. Hua and Z.P. Lei (1992). Steric Effect of the Reaction of 6,6-Dialkylfulvenes with Alkyllithium and Metal Lithium -- Synthesis of Substituted Titanocene Derivatives, Progress in Natural Science. 2: 143.

7.2 Projects as Head of Project or Project Set (หัวหน้าโครงการวิจัย) : None.

7.3 Completed Projects as Head (งานวิจัยที่ทำเสร็จแล้ว : ชื่อแผนงานวิจัย และ/หรือ โครงการวิจัย ปีที่พิมพ์ การเผยแพร่ และสถานภาพในการทำวิจัย) None

7.4 Current Projects as Head (งานวิจัยที่กำลังทำ : ชื่อแผนงานวิจัย และ/หรือ โครงการวิจัย แหล่งทุน และสถานภาพในการทำวิจัยว่าได้ทำการวิจัยลุล่วงแล้ว ประมาณร้อยละเท่าใด) None