รหัสโครงการ SUT1-102-54-12-21

# รายงานการวิจัย

# การบ่งชี้เบตากลูโคไซเดสจากข้าวที่ย่อยกลูโคไซด์ของจิบเบอริลิน

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ได้รับทุนอุดหนุนการวิจัยจาก
มหาวิทยาลัยเทคโนโลยีสุรนารี

ผลงานวิจัยเป็นความรับผิดชอบของหัวหน้าโครงการวิจัยแต่เพียงผู้เดียว

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Dr. Yanling Hua

Head of the project

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# บทคัดย่อ

สารอนุพันธ์ของจิบเบอเรลลิน (gibberellins; GA) 9 ชนิด ประกอบด้วย acetylated และ deacetylated ของ $GA_4$ glucosyl ester ($GA_4$-GE), acetylated และ deacetylated ของ $GA_3$ glucosyl ester ($GA_3$-GE), $GA_4$ และ $GA_3$ methyl ester, 3-O-β-D-glucopyranosyl $GA_3$ methyl ester, 13-O-β-D-glucopyranosyl $GA_3$ methyl ester, และ β-D-glucopyranosyl $GA_4$ methyl ester ถูกสังเคราะห์ขึ้นเพื่อใช้เป็นสับสเทรตสำหรับตรวจหาเอนไซม์เบตา-ดี-กลูโคสิเดสที่มีความสามารถในการไฮโดรไลส์สารอนุพันธ์ของจิบเบอเรลลิน โดยทำการวิเคราะห์โครงสร้างของสารที่สังเคราะห์ได้ทั้งหมดด้วยเครื่อง nuclear magnetic resonance spectroscopy (NMR) และ liquid chromatography-mass spectrometry (LC-MS)

ตัวอย่างจากเมล็ดข้าว รำข้าว ต้นอ่อน และรากข้าวที่มีอายุ 7 วัน ถูกนำมาใช้เป็นแหล่งของเอนไซม์เพื่อตรวจหาเอนไซม์เบตา-ดี-กลูโคสิเดสที่มีความสามารถในการไฮโดรไลส์สารอนุพันธ์ของจิบเบอเรลลิน โดยสกัดตัวอย่างแต่ละชนิดและแยกให้บริสุทธิ์ด้วยเทคนิค affinity chromatography และ ion exchange chromatography และตรวจหาเอนไซม์เป้าหมายที่มีความสามารถในการไฮโดรไลส์ *p*-nitophenyl β-D-glucopyranoside (*p*NPGlc) และ $GA_4$-GE จากการศึกษาพบกิจกรรมของเอนไซม์เบตา-กลูโคสิเดสในส่วนสกัดหยาบจากรำข้าว เมล็ดข้าว ต้นอ่อน และราก เช่นเดียวกันกับส่วนที่แยกได้จาก ion exchange columns อย่างไรก็ตาม ยังไม่สามารถแยกเอนไซม์บริสุทธิ์ที่มีกิจกรรมสูงได้

การทดสอบความสามารถในการไฮโดรไลส์ *p*NPGlc และ $GA_4$-GE โดยรีคอมบิแนนท์เอนไซม์ในตระกูล GH1 จำนวน 5 ชนิด ได้แก่ Os3BGlu6, Os3BGlu7 (BGlu1), Os4BGlu12, Os3BGlu18 และ Os9BGlu31 พบว่า Os3BGlu6 สามารถไฮโดรไลส์ GA4-GE ได้ดีที่สุด ในขณะที่ Os4BGlu12 สามารถไฮโดรไลส์ *p*NPGlc ดีกว่า Os3BGlu6 ถึง 50 เท่า อย่างไรก็ตาม Os4BGlu12 สามารถไฮโดรไลส์ GA4-GE ได้เพียงร้อยละ 20 ของที่ไฮโดรไลส์โดย Os3BGlu6 จากข้อมูลที่ได้ Os3BGlu6 จึงถูกใช้เป็นต้นแบบ ในการศึกษาการไฮโดรไลส์ glucosyl ester (GA4-GE) เปรียบเทียบกับการไฮโดรไลส์กลูโคไซส์

## Abstract

In order to monitor the extraction of β-D-glucosidases that can hydrolyze gibberellin (GA) conjugates from rice, 9 GA conjugates, which included acetylated and deacetylated gibberellin $GA_4$ glucosyl esters ($GA_4$-GE), acetylated and deacetylated $GA_3$ glucosyl esters, $GA_4$ methyl ester, $GA_3$ methyl ester, 3-O-β-D-glucopyranosyl gibberellin $A_3$ methyl ester, 13-O-β-D-glucopyranosyl gibberellin $A_3$ methyl ester and β-D-gluco-pyranosyl gibberellin $A_4$ methyl ester, were synthesized. Their structures were identified with nuclear magnetic resonance spectroscopy (NMR) and liquid chromatography-mass spectrometry (LC-MS).

To identify a GA β-D-glucosidase from rice, rice seeds, glumes, 7-day rice seedlings and roots were extracted and the β-D-glucosidase activities were purified with affinity chromatography and ion exchange chromatography. The fractions were monitored for hydrolysis of *p*-nitrophenyl β-D-glucopyranoside (*p*NPGlc) and $GA_4$-GE. The β-glucosidase activities were found in the crude extracts of glumes, seeds, seedlings and roots, as well as their fractions from the ion exchange columns; but the pure protein with high activity has not been separated yet.

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. Based on these data, Os3BGlu6 has become a model for hydrolysis of the glucosyl ester ($GA_4$-GE) in comparison to glucosides.

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**Section 2**

# CHAPTER 1. Introduction

## 1.1. Importance and Background of Reseach Problem (ความสำคัญและที่มาของปัญหาการวิจัย)

***Background and literature survey***

### 1.1.1 Rice and Rice Gene Evaluation

Rice (*Oryza sativa* L.) is the second most highly produced crop in the word, after corn (Zea mays), and the most important food crop in the world, with the second being its fellow Grameaceae wheat (*Triticum aestivum* L.). Since it has a relatively small genome, rice was designated as the first monocot to have its genome sequence and the genome was sequenced to near completeness [International Rice Genome Project, 2005]. This, along with efforts to produce expressed sequence tags (ESTs) 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]. An assortment of microarray experiments have also been done with much of the data obtained available in public data bases [Rensink and Buell, 2005]. Thus, rice provides a wealth of research for investigation of important processes in plants, particularly monocots, which include many of the world's most important crops.

Aside from the sequence-related 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]. The rice gene insertion lines can often be identified by a search of publicly available databases, and the functions of the disrupted genes can then be investigated by ordering and growing the lines or by collaboration with those in 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]. These gibberellins have been found to be 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 they derive their name. The presence of large quantities of GAs as secondary metabolites in this fungus leads to the extensive overgrowth of infected rice plants. Gibberellic acid ($GA_3$), which was the first gibberellin to be structurally characterized, has been widely used to regulate the plant growth and development. The pathway of gibberellin synthesis in the plant has been characterized and many of the genes for the synthetic enzymes characterized. Their 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].

The gibberellins are classified on the basis of structure as well as function. Naturally occurring tetracyclic diterpenoid acids with structures based on the ent-gibberellane carbon skeleton (Fig.1.1) are named $GA_1$ to $GA_n$ in the order of their discovery (www. plant-hormone.info /gibberellins).

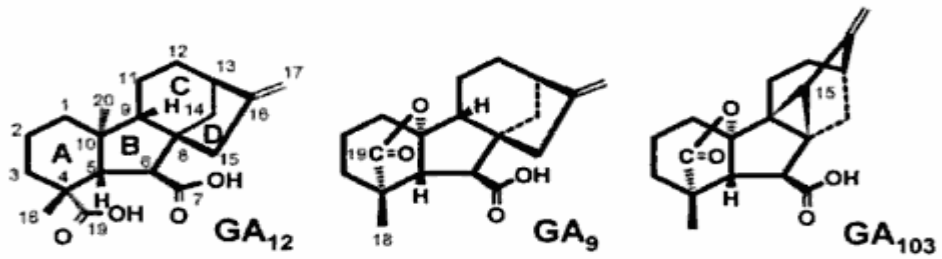



**Figure 1.1** Structures of gibberellins with different levels of structural complexity. $GA_{12}$ is a C20-GA with the ent-gibberellane skeleton; $GA_9$ is the simplest C19-GA, which has an ent-20-norgibberellane skeleton, and $GA_{103}$, has an extra cyclopropane ring [Sponsel and Hedden, 2004].

Bioactive gibberellins exist in the 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, each depending on the type of gibberellin present as well as the species of plant. Gibberellins have been found to be involved in many physiological processes [Davies, 1995; Mauseth, 1991; Raven, 1992; Salisbury and Ross, 1992]. They

stimulate stem elongation by stimulating cell division and 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 for mobilization of seed reserves. Gibberellins also induce maleness in dioecious flowers (sex expression), can be used to cause parthenocarpic (seedless) fruit development and delay senescence in leaves and citrus fruits.

**1.1.2.2. Gibberellin conjugates**

Gibberellins exist in plants both as the free acids and in conjugated forms. After the isolation and identification of the first gibberelin from higher plants -$GA_1$ [MacMillan et al., 1958], many GA-conjugates were discovered. 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]. After that, 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]. Today, the conjugation process is considered to be an important aspect of GA metabolism in plants. 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.

The most common GA conjugates isolated from plants are GA glucosyl conjugates, in which the GAs are connected to glucose. 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 esters 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.

Upon conjugation of gibberellins to GA glucosyl conjugates, the biological activity is reduced or totally lost, but the increased polarities of GA glucosyl conjugates are considered to 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 tend to form and accumulate during the seed maturation period, it has been proposed that this group of compounds may 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 over a 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. 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 decreased 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

Glycoside hydrolases (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 and Baroch 1993, 1996]. The latter approach is amenable to analysis of large amounts of sequence data that has become available with genomic sequencing projects and is used to give a clue to the functions of various gene products [International Rice Genome Project, 2005]. However, this practice 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 (and sometimes even their basic functions). Nonetheless, 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, more than one hundred 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 and Henrissat, 1999; Cantarel et al., 2009]. Despite their similar sequences and mechanisms, members of GH families generally include enzymes with a few to several different substrate specificities. For instance, GH family 2 (GH2) contains β-galactosidases, β-glucuronidases and β-mannosidases, while GH3 contains exoglucanases and β-xylosidases (CAZY).

#### 1.1.3.1. Clan A beta-glycosidases

Clan A β-glycosidases have catalytic domains that form $(\beta/\alpha)_8$-barrels, 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 [Jenkins et al., 1995; Henrissat et al., 1995; Chuenchor et al., 2008]. As such, they generally catalyze hydrolysis by a shared retaining mechanism in which the stereochemistry of the anomeric carbon in the released sugar is initially 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. This structure and mechanism appear to be very successful for hydrolysis of glycosides and carbohydrates

as 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, although certain of the enzymes 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, for example].

#### 1.1.3.2. Functions of plant β-glucosidases

We have previously annotated the enzymes of GH1 and GH35 in rice to begin to unravel the functions of these β-D-glycosidases in plants [Opassiri et al., 2006; Tantanuch et al., 2008]. In general, 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 made up of a large assemblage of cellulose, hemicelluloses, pectins and related polysaccharides, glycoproteins, and lignins, as well as silica and other components, depending on the type of cell wall [Carpita, 1996; Cosgrove, 1997]. During growth and development, plants constantly remodel and turnover their cell wall polysaccharides, and in processes like breakdown of endosperm cell walls in germination, 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 glucose and other sugar 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. Thus, plants generally

have a wide range of enzymes with different specificities to act at different points in cell wall degradation and recycling.

Many GH1 enzymes from plants hydrolyze β-linked oligosaccharides, suggesting this may be an important role for them in the plant [Akiyama et al., 1998; Opassiri et al., 2003, 2004, 2006; Kuntothom et al., 2009]. The rice BGlu1 (Os3BGlu7), Os3BGlu8 and Os7BGlu26 isoenzymes and a closely related enzyme from barley [Hrmova et al., 1996, 1998, 2006] can also hydrolyze β-linked mannooligossacharides, suggesting they could have roles in recycling of multiple cell wall components. A similar enzyme is found in Arabidopsis [Xu et al., 2004], so this kind of plant exoglycosidase may be wide spread.

Plant β-glucosidases also act in the development of secondary cell walls by releasing lignin precursors at sites of lignin formation [Dharmawardhana et al., 1995]. The presence of monolignol glucoside β-glucosidases has been shown in Arabidopsis [Escamilla-Treviño et al., 2006], as well as pine trees, and three closely related genes and two gene fragments are found in the rice genome [Opassiri et al., 2006]. This implies that β-glucosidases may modulate the mechanical properties and digestibility of cell wall by affecting lignin deposition, in addition to their roles in polysaccharide recycling. Decreasing lignin content is one of the main goals of genetic engineering of plants for bioconversion to fuels [Sticklen, 2006], which makes the effect of these β-glucosidases on cell wall lignin content especially interesting.

**1.1.3.2.2. Defense**

A large number of β-glucosidases have been shown to release toxic compounds from inactive glycosides in response to herbivory, fungal invasion and damage [Morant et al., 2008]. Cyanogenic glycosides, which rapidly react to release HCN upon release from their glucosyl blocking group by β-glucosidases [Poulton, 1990]. As noted above, hydroxaminic acids, like DIMBOA and DIBOA can be released from their glucosides in maize [Babcock and Esen, 1994], wheat and rye [Sue et al., 2006] by β-glucosidases. 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.

The 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 sometimes appear to 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]. In many eudicots, cyanogenic β-glucosidases are found in the apoplast, while the substrates are found in the vacuole [Morant et al., 2008 and references therein]. Aside from localization, another common feature of defensive β-glucosidases is the presence of a cytoplasmic aggregating factor that binds to them upon cell wall disruption [Blanchard et al., 2001]. These proteins are thought to affect the localization after the cells are broken open to make the β-glucosidases most effective against the herbivore or pathogen.

**1.1.3.2.3. Phytohormone activation**

The roles of phytohormone glycosides and that of their hydrolysis by β-glycosidases (β-glucosidases) has been debated [Buchanan et al., 2000]. Several of these glycosides have been suggested to be deadend inactivation products of the phytohormones, although their high levels and availability suggest that they could be a source for rapid release of phytohormones in stress responses and development. Enzymes have been identified that can hydrolyze glycosides of gibberrilins [Schliemann, 1984], cytokinin [Brzobohatý et al., 1993], auxin [Jakubowska & Kowalczyk, 2005], jasmonic acid derivatives [Seto et al., 2009; Wakuta et al., 2010], and abscissic acid [Lee et al., 2006]. 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].

#### 1.1.3.2.4. Response to abiotic and biotic stress

In many cases β-glucosidases have been noted to be increased in response to abiotic or biotic stress, although their roles are often unknown. As mentioned above, 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]. Moreover, Arabidopsis plants mutant in this gene were impaired in the ability to close stomata in response to drought. It was found that the AtBG1 was aggregated 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. Although this is a fairly well defined mechanism of β-glucosidase action in response to stress, the mechanisms of other β-glucosidases are not so clear. A β-glucosidase has been identified from an Arabidopsis mutant *sensitive to freezing 2 (sfr-2*), in which the plants could not recover from cold stress [Thorlby et al., 2004]. The enzyme is found in the chloroplast and seems to be distantly related to other plant GH1 β-glucosidases, but its substrates were unknown until it was recently found to be a galactolipid galactolipid galactosyl transferase (GGGT) involved in chloroplast membrane recycling [Moellering et al., 2010]. Other β-glucosidases have been found to be upregulated in responses to stresses like 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

Schliemann [1984] first reported characterization of dwarf rice seedling β-glucosidases that could hydrolyze synthetic glucoconjugates. However, the β-glucosidases were apparently only partially purified and no molecular characterization was done. Akiyama and colleagues [1998] purified a cell-wall-bound β-glucosidase from rice seedlings and showed that it was active against cell-wall-derived oligosaccharides, as well as determining the amino-terminal amino acid sequence. 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 had 6 subsites for binding β-(1,4)-linked gluco-oligosaccharides and had 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 (designated *Os3bglu7*, based on its chromosomal location) was most abundantly expressed. Subsequently it was found that other isoenzymes in the same phylogenetic cluster as Os3BGlu7, Os3BGlu8 and Os7BGlu26, also hydrolyzed 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 [Opassiri et al., 2006]. However, further it was found to hydrolyze *p*-nitrophenyl glycosides more efficiently and also hydrolyze steroid and flavonoid beta-glucosides with similar efficiency [Opassiri et al., 2010]. Recently, it was found to efficiently hydrolyze salicylic acid glucoside (SAG) [Himeno et al., 2013]. 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 and Os4BGlu12 have been determined and it can be seen that small differences in their active sites can account for their differences substrate specificities [Chuenchor et al., 2008; Seshadri et al., 2009; Sansenya et al., 2011].

It is worth considering that 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. Recently, the isoenzyme most closelty related to Os4BGlu12 (Os4BGlu13) was found to hydrolyze tuberonic acid beta-glucoside (TAG), and was designated TAG beta-glucosidase I (TAGG1) [Wakuta 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 Recombinant expression of rice beta-glucosidases previously cloned in our laboratory and testing whether they hydrolyze gibberellin glucoconjugates.

### 1.3. Scope of Research (ขอบเขตของการวิจัย)

The scope of this project was to synthesize the gibberellin $GA_3$ and $GA_4$ beta-glucoconjugates and their use for beta-glucosidase identification by 1) testing purification fractions of rice bran extracts and 2) testing the seven or eight rice enzymes with β-glucosidase activity that have been cloned and expressed in our laboratory. In this project, the $GA_3$ and $GA_4$ glucosyl ester and $GA_3$ and $GA_4$ glucoside were synthesized. The beta-glucosidases that hydrolyze $GA_4$ glucosyl ester were extracted from rice bran, glumes, 7-day rice seedlings and roots, and purified with chromatography. Proteins that are predominant in those fractions were identified by tryptic digest and mass spectrometry of sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE) bands and comparison of the peptide masses to predicted proteins from the rice genome database. In addition, the proteins that have previously been cloned and expressed in our laboratory, Os3BGlu6, Os3BGlu7, Os4BGlu12, Os3BGlu18 and Os9BGlu31, were tested for hydrolysis of the $GA_4$ glucoconjugates.

### 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 $Ag_2O$ 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' activities 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 1 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 was 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 the previously 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, different rice tissues 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 ConA sepharose column, which was eluted with a step gradient of mannose in McIlvaine buffer. The separation of the β-glucosidases was monitored with *para*-nitrophenol β-D-glucopyranoside (*p*NPGlc) and GA-glucosyl conjugates as substrates. The dialysed enzyme extract was clarified by short centrifugation and the soluble enzyme fraction was fractioned by Q-sepharose or a similar ion exchange column. Those fractions containing the protein of interest were separated on SDS-PAGE and the 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.

**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 in the journal **Arch. Bichem. Biophys.** [Hua, Y., Sansenya, S., Saetang, C., Wakuta, S. and Ketudat Cairns, J. R. (2013) Enzymatic and structural characterization of hydrolysis of gibberellin A4 glucosyl ester by a rice β-D-glucosidase].

# CHAPTER 2. Materials and Methods

## 2.1 Materials

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 ($Ag_2O$), 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). The 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 following the method of Hiraga et al. (1974, Figure 2.1). Briefly, 3 mmol of gibberellin $GA_3$ or $GA_4$ was dissolved in 50 ml of dry dioxane (dried overnight with molecular sieves 4Å) in a 100 ml round bottom flask with three necks. Then, 3 mmol of α-acetobromo-glucose and 3 mmol silver oxide ($Ag_2O$) were added to the solution under nitrogen gas ($N_2$) flushing. The mixture was stirred overnight at room temperature in darkness. Solids was filtered and washed with ethyl acetate (EtOAc) two times. The filtrate was extracted twice with 10% (w/v) of sodium bicarbonate ($NaHCO_3$). The organic phase was separated from the aqueous phase, dried with anhydrous sodium sulphate ($Na_2SO_4$, dried in the oven before use) and evaporated with a rotary evaporator at 40°C to get a solid. The solid was purified with ethyl acetate-hexane to get a white powder. Acetylated $GA_4$-GE was produced with 43.7% yield (0.870 g), while acetylated $GA_3$-GE was obtained in 18.3% yield.

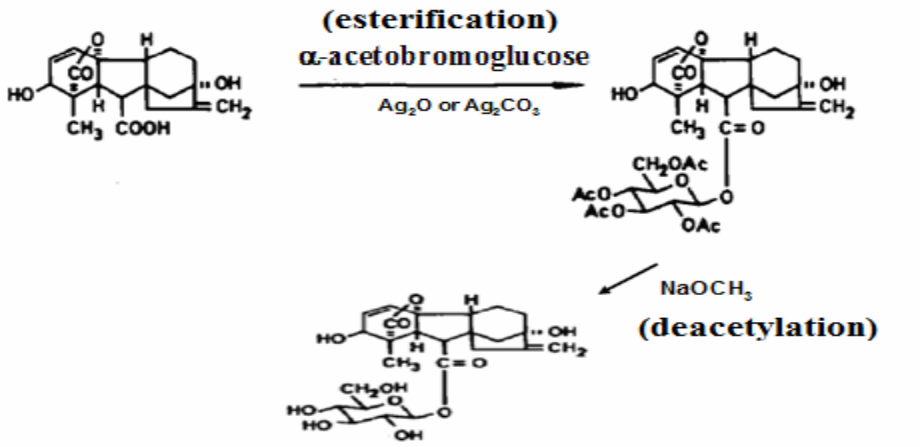



**Figure 2.1** Reaction scheme for synthesis of gibberellin glucosyl esters (Hiraga et al., 1974).

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 Synthesis of gibberellin methyl esters of $GA_3$ and $GA_4$

Synthesis of the glucosides was begun with methyl esterification of the carboxyl groups of $GA_3$ and $GA_4$. The methylation of $GA_3$ and $GA_4$ followed the procedures of Lombardi [1990] (Figure 2.2) and was performed in the device shown in Figure 2.3.

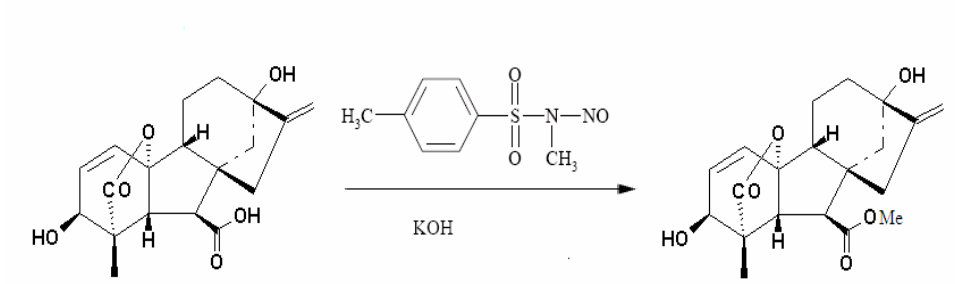



**Figure 2.2** Reaction scheme for synthesis of gibberellin methyl esters.

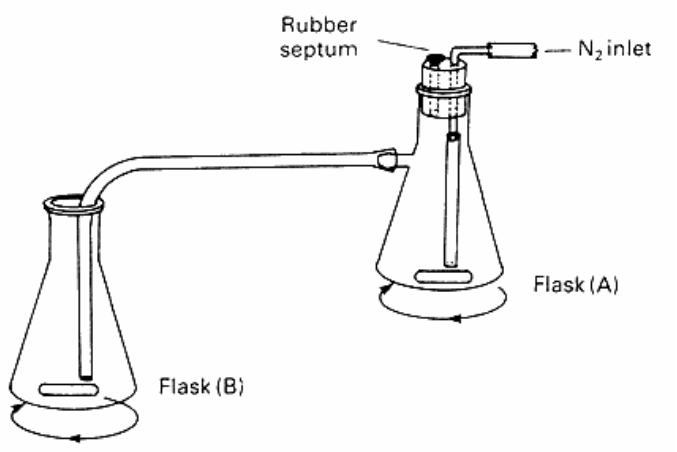



**Figure 2.3** The device for methylation reaction [Lombardi, 1990).

In flask B, $GA_3$ or $GA_4$ (5 mmol) was dissolved in methylene dichloride ($CH_2Cl_2$, 40 ml) and ethanol (1 ml), and cooled to 0°C with stirring. *p*-Toluenesulphonylmethyl-nitrosamide (Diazald, 7.5 mmol) was suspended in ethanol (20 ml) in flask A, and the mixture was stirred while nitrogen was

allowed to flow into the flask. Sodium hydroxide solution (30%, w/v) was added dropwise to the flask A. After a few seconds, yellow diazomethane began to evolve in flask A and passed into flask B where reaction with the substrate occurred. Sodium hydroxide solution was added dropwise to flask A at a rate of 2 ml/s until the yellow color in flask A was discharged. The solution in flask B was monitored by TLC using hexane/EtOAc (9:11, v:v) to check the completion of the methylation reaction.

The solvent was removed in a rotary evaporator to get the crude methylation products. The crude products were purified by Solid Phase Extraction (SPE) in an SPE manifold (MACHEREY-NAGEL). The aminopropyl modified silica cartridge-Chromabond® $NH_2$ (3 ml, MACHEREY-NAGEL) was rinsed with 3 ml of EtOAc, then 3 ml of crude sample in EtOAc was loaded onto the cartridge, and the flow-through was collected. The cartridge was washed with 3 ml of hexane-EtOAc (1:1, v:v), 3 ml of EtOAc and 3 ml of MeOH, respectively; and the fractions were collected. The fractions were checked with TLC, the fractions with $GA_3$-OMe or $GA_4$-OMe were combined, and the solvents removed by TurboVap® LV to get white power. The yields for $GA_3$-OMe and $GA_4$-OMe were 90.0% and 67.4%, respectively.

### 2.2.1.3 Synthesis of β-D-glucopyranosyl gibberellin methyl esters of $GA_3$ and $GA_4$

β-D-Glucopyranosyl gibberellin methyl esters of $GA_3$ and $GA_4$ were synthesized following the method of Scheiber et al. [1969] (Figure 2.4).

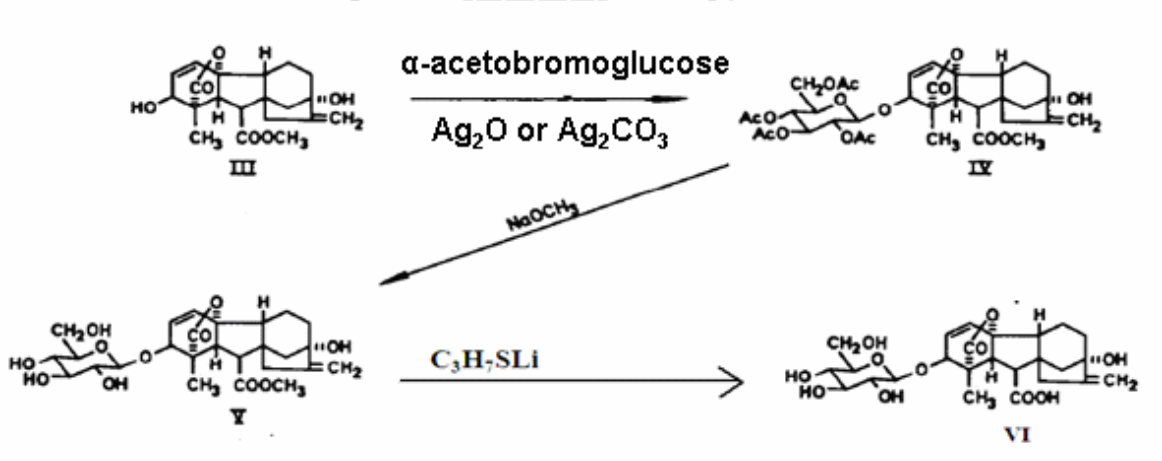



**Figure 2.4** Reaction scheme for synthesis of β-D-glucopyranosyl gibberellin methyl ester [Scheiber et al., 1969].

One millimole of $GA_3$-OMe or $GA_4$-OMe, 3 ml of dry dioxane and 1.4 mmol of $Ag_2O$ were added into a 100 ml round bottom flask with three necks. Then, 1.2 mmol of α-acetobromoglucose was dissolved in 10 ml of dry benzene and added to the flask dropwise under nitrogen gas ($N_2$) flushing. The flask was heated and 5 ml of benzene was distilled out. Then, the reaction solution was stirred at room temperature for 72 h. Forty milliliters of EtOAc was added into the reaction mix to dissolve the solid; the undissolvable solid was filtered and washed with EtOAc. The solvent from the filtrate was removed by evacuation in a rotary evaporator to get crude product. The crude product was then purified by flash column chromatograph over silica gel with hexane/EtOAc to get pure product. The yield for tetra acetylated β-D-glucopyranosyl gibberellin $A_3$ methyl ester ($GA_3$-OMe-Glc-$Ac_4$) was 54.3%, and for tetra acetylated β-D-glucopyranosyl gibberellin $A_4$ methyl ester ($GA_4$-OMe-Glc-$Ac_4$) was 50.0%.

The deacetylation followed the method described in section 2.2.1.1. The yields for β-D-glucopyranosyl gibberellin $A_3$ methyl ester ($GA_3$-OMe-Glc) and for β-D-gluco-pyranosyl gibberellin $A_4$ methyl ester ($GA_4$-OMe-Glc) were approximately 10%.

#### 2.2.1.4 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 rice tissues**

Rice seeds were ground to powder in liquid $N_2$, 7-day rice seedlings and roots were cut to small pieces with a blender before extraction. Rice seeds, glumes, 7-day rice seedlings and roots were 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 (buffer A), 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 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 20 mM Tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl), pH 7, containing 0.5 M NaCl (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 (buffer C), before testing activity with $GA_4$-GE and further purification by gel filtration chromatography.

**2.2.2.3 Purification of β-glucosidase by ion exchange chromatography with a HiTrap Q-Sepharose column**

A HiTrap Q-Sepharose column (5 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, and eluted with a linear gradient of 0-1.0 M NaCl in buffer C 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 in sodium dodecyl sulfate (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 5.5 (buffer D).

**2.2.2.4 Purification of β-glucosidase by ion exchange chromatography with a 2rd HiTrap Q-Sepharose column**

A HiTrap Q-Sepharose column (5 ml, GE Healthcare) was equilibrated with 2 CV of buffer D on an ÄKTA Protein Purifier system (GE Healthcare). Three milliliters of concentrated protein from HiTrap Q-Sepharose column was loaded to the column with a super loop, and eluted with a linear gradient of 0-1.0 M NaCl in buffer D 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 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 5.5 (buffer D).

**2.2.2.5 Purification of β-glucosidase by ion exchange chromatography with a 3$^{rd}$ HiTrap Q-Sepharose column**

A 1 ml HiTrap Q-Sepharose column (GE Healthcare) was equilibrated with buffer D on an ÄKTA Protein Purifier system. Five hundred microliters of concentrated protein from the 2rd HiTrap Q-Sepharose 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 D 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.

**2.2.2.6 Identification of protein bands with LC-MS**

The protein from the HiTrap Q-Sepharose column purification was separated on an 8% SDS-PAGE. The 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 4°C 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). 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 column temperature was maintained at 35°C. Analysis of tryptic peptides was performed on a SYNAPT$^{TM}$ HDMS mass spectrometer (Waters Corp., Manchester, UK). All analyses were performed in positive ion nanoelectrospray mode. 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.

**2.2.2.7 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 in the same buffer solution.

The hydrolysis of $GA_4$-glucosyl ester ($GA_4$-GE) was determined with a peroxidase/glucose oxidase-based assay (PGO assay, Sigma). 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 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) were added to the reaction mixes, 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.

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

SDS-PAGE was performed according to Laemmli [1970]. 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 in a water bath, centrifuged 2 minutes, and then loaded onto the gel. The protein standard marker was loaded on to the same gel. The electrophoresis was run at 200 V for 50 min in 1X running buffer (0.3% (w/v) Tris-base, 1.4% (w/v) glycine, 1% (w/v) SDS). The gel was stained with Coomassie brilliant blue R-250 (CBB) solution for 40 min, and then destained with destaining solution (40% methanol and 7% acetic acid) twice, followed by water. The gel was dried at 55°C for 90 min under vacuum.

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], Os3BGlu18 (Baiya et al., unpublished) and Os9BGlu31 [Luang et al., 2013] were tested for the hydrolysis activity to *p*NPGlc and $GA_4$-Glc according to method described in section 2.2.2.7.

# 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 (Figure 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 $^1$H NMR was consistent with the published data for $GA_4$-GE [Hiraga et al., 1974].

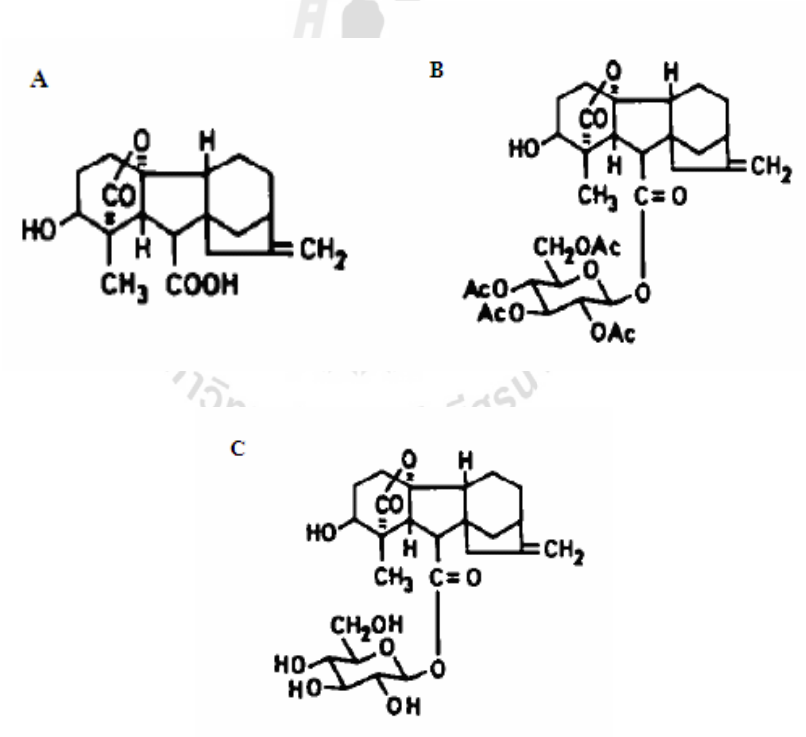



**Figure 3.1** The structures of the gibberellin $GA_4$ and its derivatives. **A**. $GA_4$, $C_{19}H_{24}O_5$, molecular weight 332.39; **B**. Acetylated $GA_4$-GE, $C_{33}H_{42}O_{14}$, molecular weight 662.68; **C**. $GA_4$-GE, $C_{25}H_{34}O_{10}$, molecular weight 494.53.

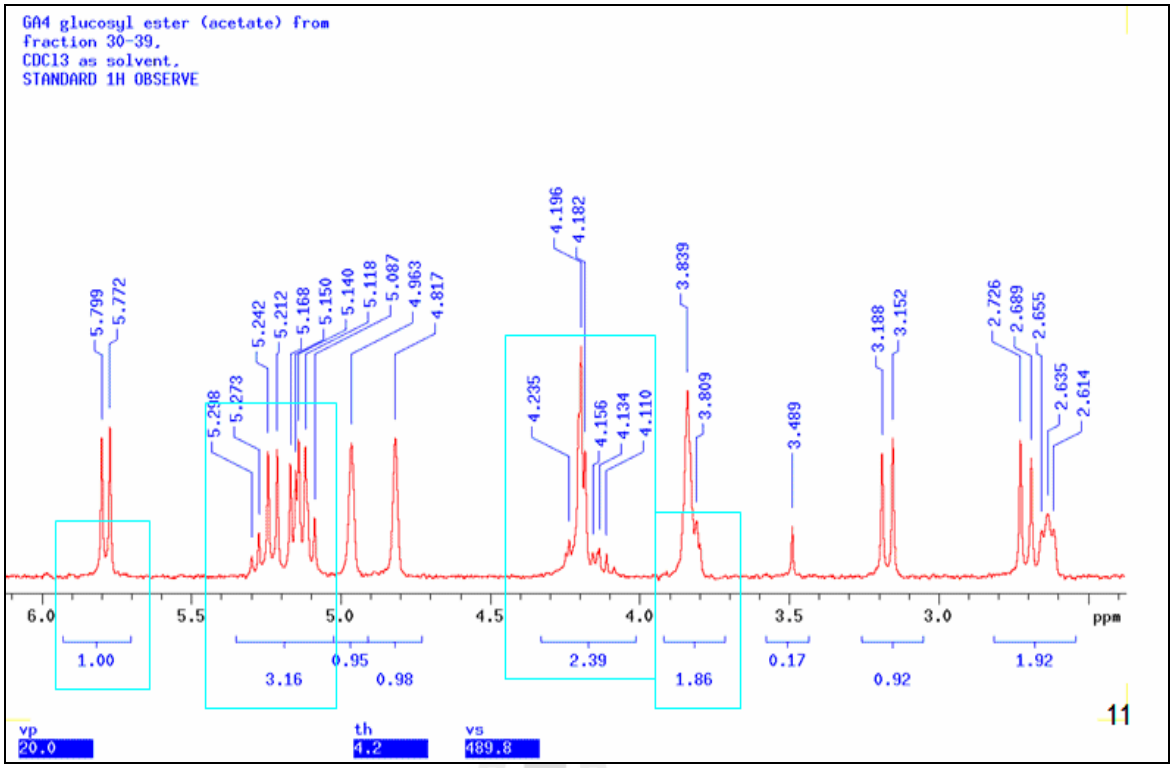



**Figure 3.2** Expanded view of the $^{1}$H NMR spectrum of acetylated $GA_4$-GE in $CDCl_3$. The 7 protons on the glucosyl ring are boxed in rectangles (one proton from $GA_4$ was included at position δ3.839). The coupling constant of the H1 peak at δ5.786 ($J_{2,1}$=8.1 Hz) confirmed that the acetylated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

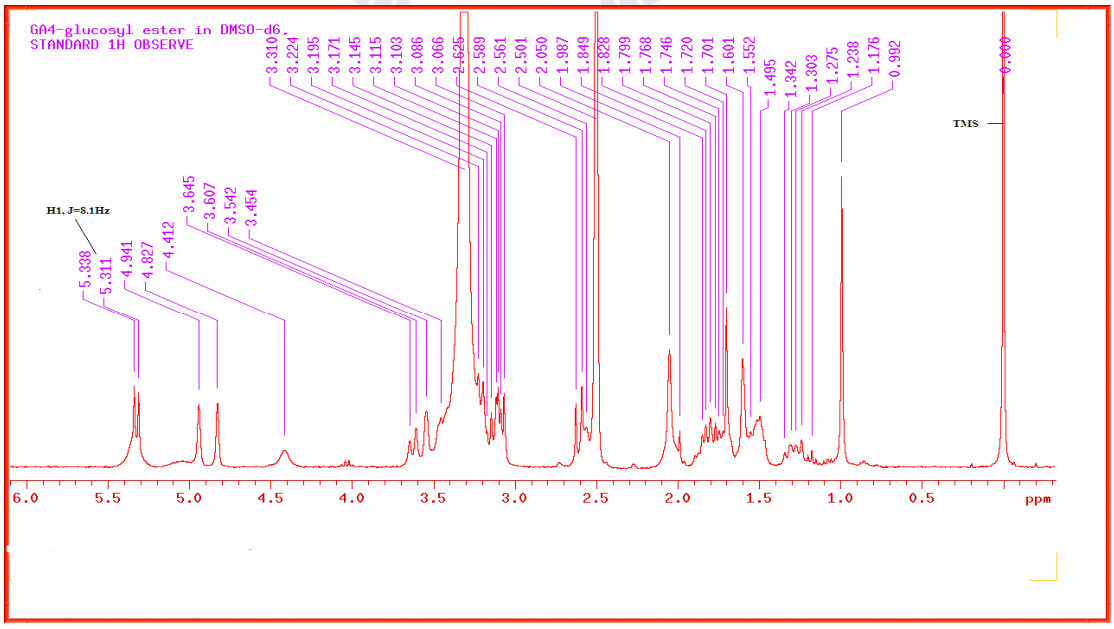



**Figure 3.3** $^{1}$H NMR spectrum of $GA_4$-GE in DMSO-d6 with TMS as reference standard. δ5.32 was assigned as H1, d, $J_{2,1}$=8.1 Hz, which confirmed that the $GA_4$-GE had a β-configuration.

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.

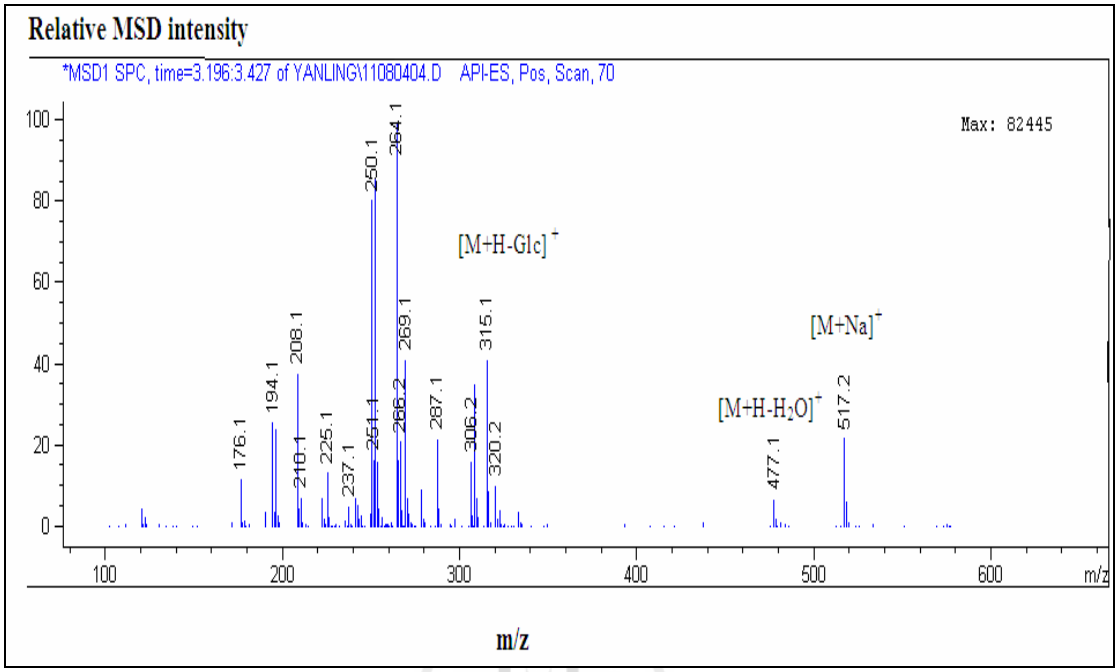



**Figure 3.4** The mass spectrum of $GA_4$-GE in the positive mode. $[M+Na]^+$, $[M+H-H_2O]^+$ and $[M+H-Glc]^+$ are marked on the mass spectrum.

### 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.

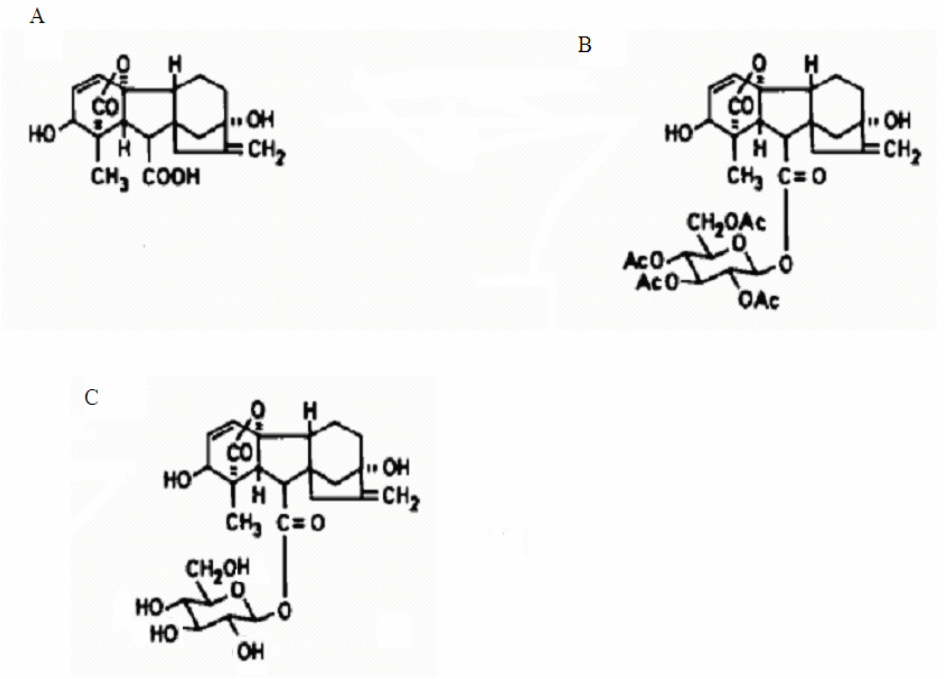



**Figure 3.5** The structures of the gibberellin $GA_3$ and its derivatives. **A**. $GA_3$, $C_{19}H_{22}O_{6,}$ molecular weight 346.37; **B**. Acetylated $GA_3$-GE, $C_{33}H_{40}O_{15}$, molecular weight 676.36; **C**. $GA_3$-GE, $C_{25}H_{32}O_{11}$, molecular weight 508.51.

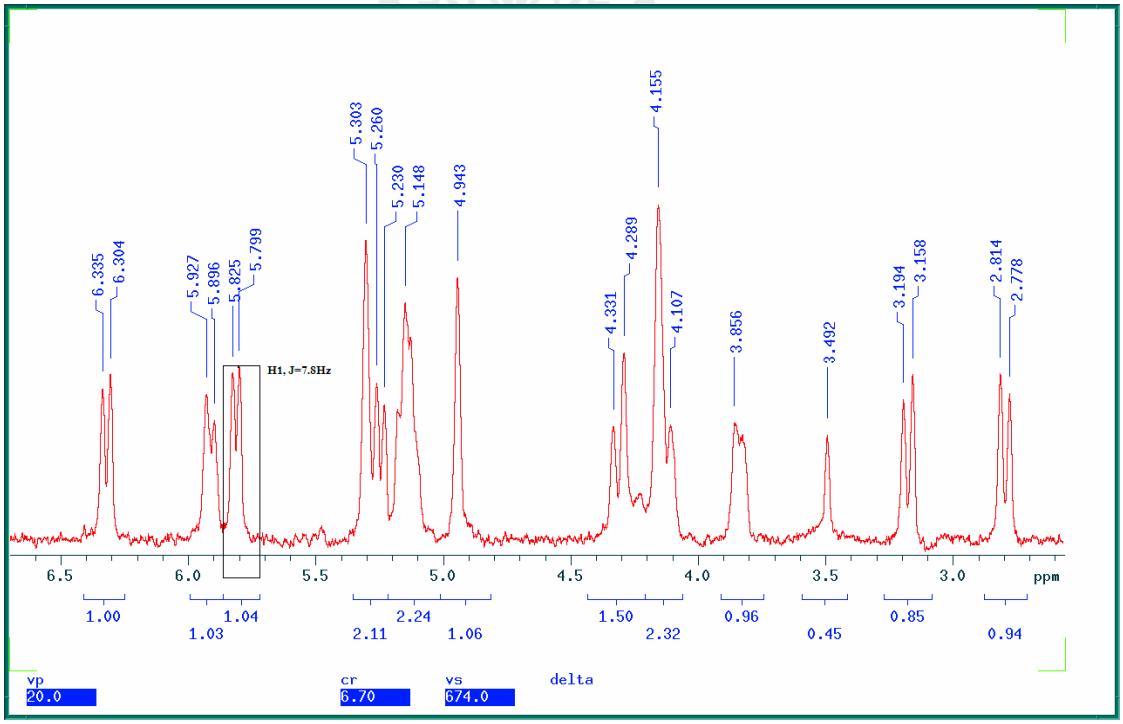



**Figure 3.6** The expanded $^1H$ NMR spectra of the acetylated $GA_3$-GE in $CDCl_3$. TMS was the reference standard. The peak for the anomeric H1 proton on the glucosyl ring is enclosed in the box.

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 $^1H$ NMR of $GA_3$-GE was also consistent with the published data (Hiraga et al., 1974).

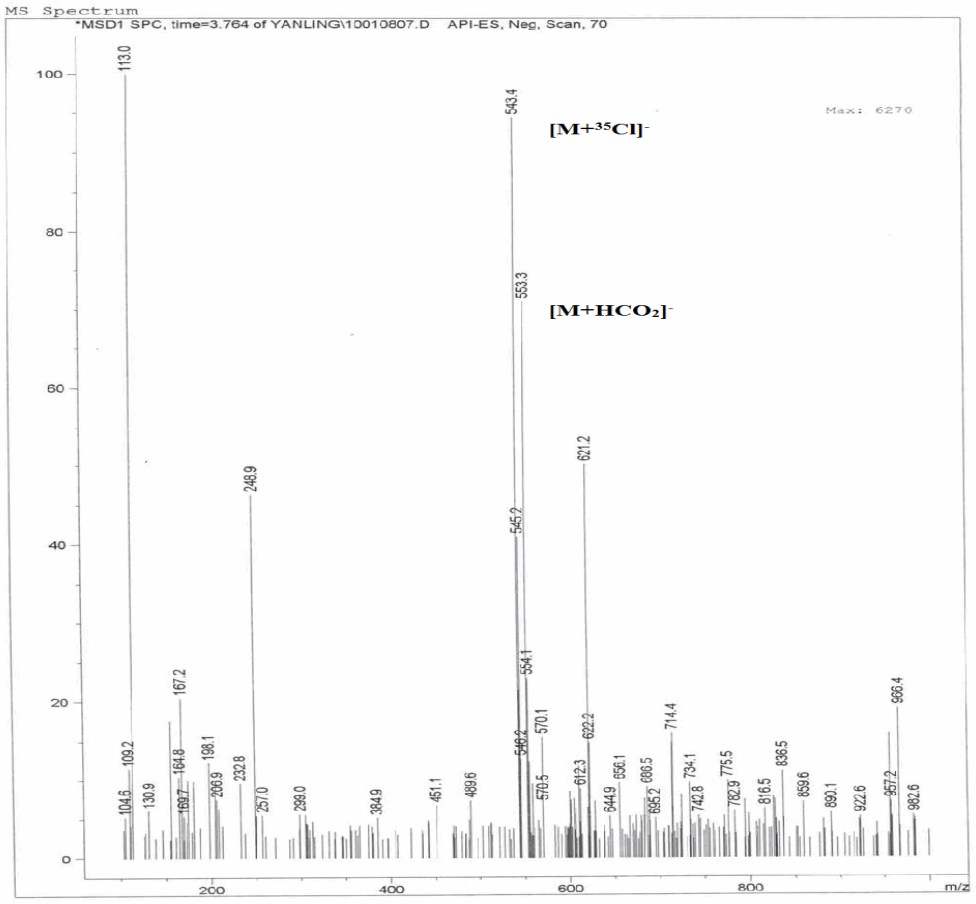



**Figure 3.7** The mass spectrum of $GA_3$-GE in the negative mode. Peaks for $[M+^{35}Cl]^-$ at *m/z* 543.4, $[M+^{37}Cl]^-$ at *m/z* 545.2 and $[M+HCO_2]^-$ at *m/z* 553.3 are found in the mass spectrum.

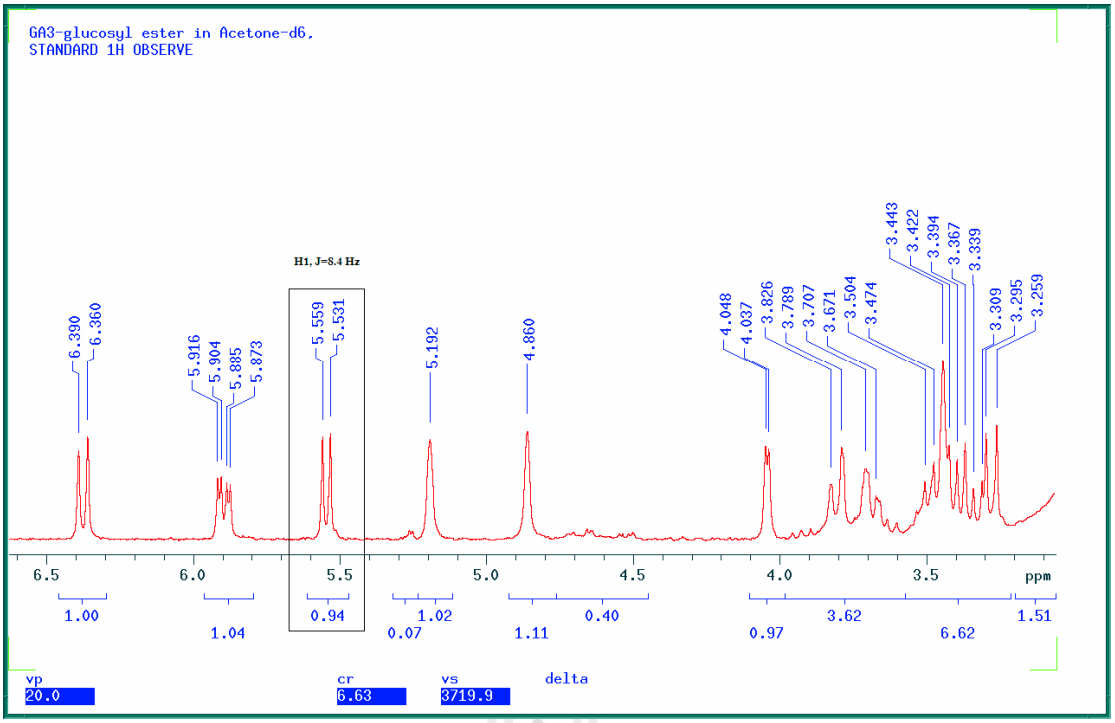



**Figure 3.8** The expanded (bottom) $^{1}$H NMR spectra of the $GA_3$-GE in acetone-d6. The doublet for the H1 proton on the glucosyl ring is boxed in the rectangle.

### 3.1.3 Synthesis of gibberellin $GA_3$ methyl ester and its glucosides

$GA_3$-OMe and its two glucosides (Figure 3.9) were synthesized and identified with mass and NMR spectrometry as described in section 2. $GA_3$-OMe was confirmed from its mass spectrum (Figure 3.10). In the positive mode, $[M+Na]^+$ was found as the base peak at *m/z* 383.3, $[2M+Na]^+$ was found at *m/z* 743.5. The $^{1}$H NMR spectrum (Figure 3.11) was acquired for $GA_3$-OMe for comparison.

The two $GA_3$-OMe glucosides were separated by LC-MS (Figure 3.12), and their molecular masses were confirmed in their mass spectra (Figure 3.13). $[M+HCO_2]^-$ were found as the base peaks, *m/z* at 567.2, for both of them, although these two glucosides had different fragment patterns under the same ionization conditions.

The $^{1}$H NMR spectra were collected for these two glucosides, and they were compared with the spectrum of $GA_3$-OMe (Figure 3.14). The differences were seen mainly for H2 and H17 on the skeleton of $GA_3$. HPLC peak 2 was assigned as $GA_3$-OMe-3-O-Glc, since its chemical shift value for the peak of H2 was higher than the one on $GA_3$-OMe and $GA_3$-OMe-13-O-Glc, because of the glucosyl linkage at carbon position 3.

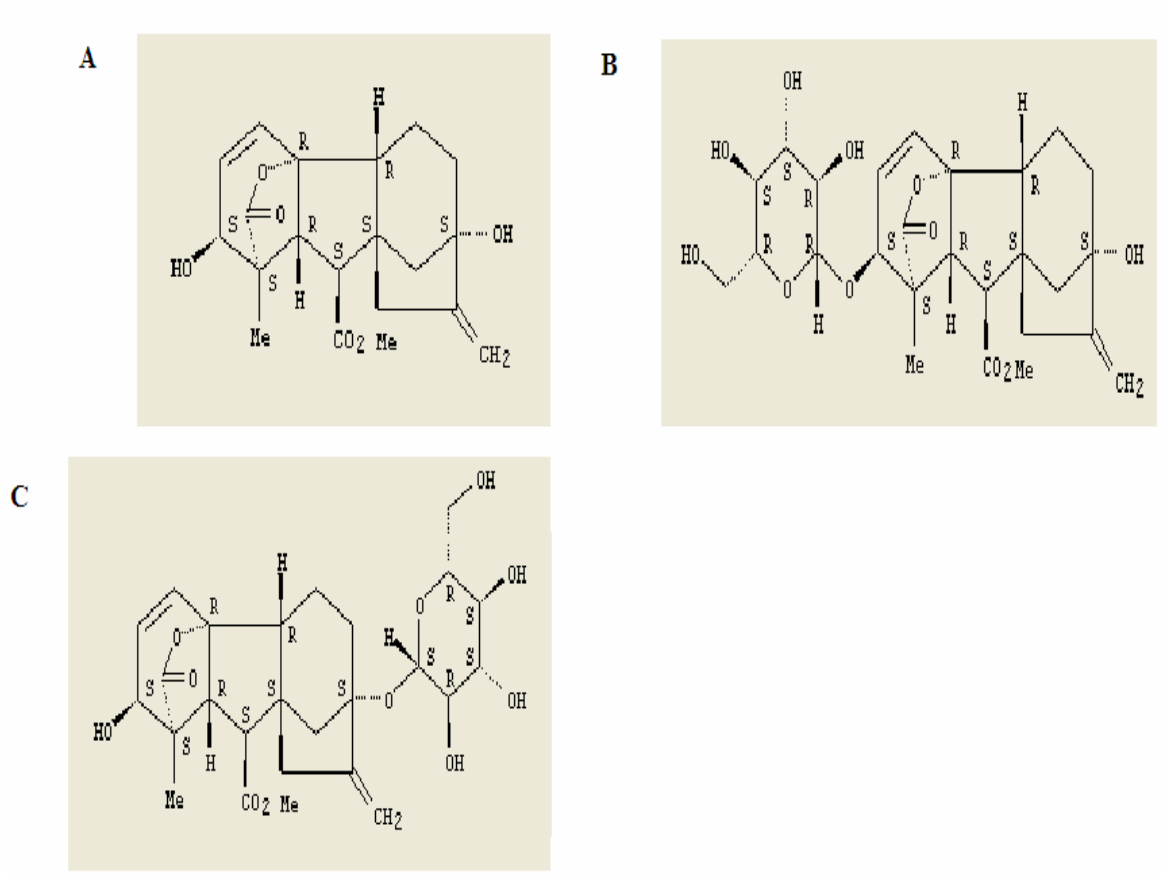



**Figure 3.9** The structures of the $GA_3$-OMe (A), $GA_3$-OMe-3-O-Glc (B) and $GA_3$-OMe-13-O-Glc (C). **A**. $GA_3$-OMe, $C_{20}H_{24}O_{6,}$ molecular weight 360.40; **B**. $GA_3$-OMe-3-O-Glc, $C_{26}H_{34}O_{11}$, molecular weight 522.54; **C**, $GA_3$-OMe-13-O-Glc, $C_{26}H_{34}O_{11}$, molecular weight 522.54.

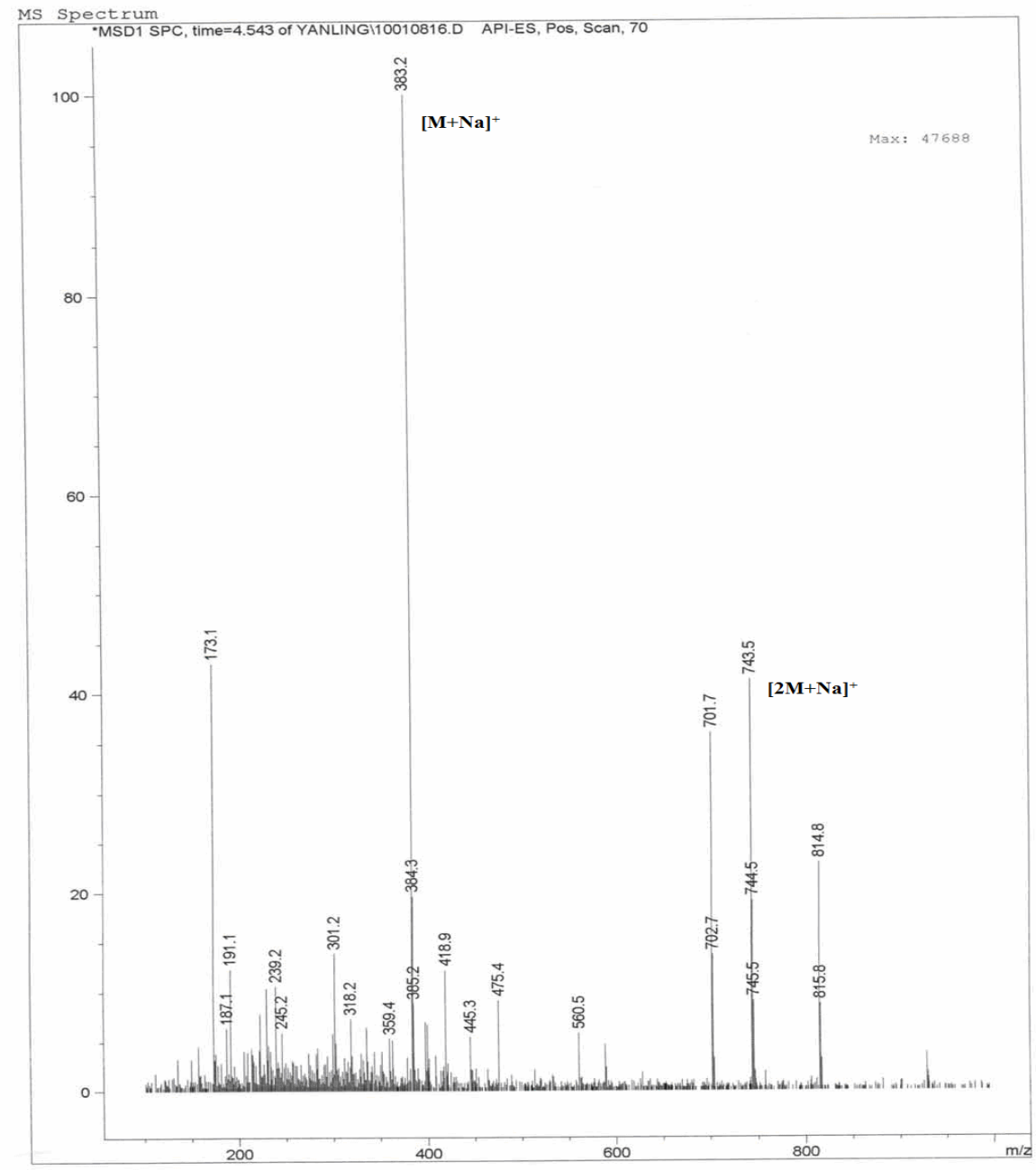



**Figure 3.10** The mass spectrum of $GA_3$-OMe. The peak for $[M+Na]^+$ is found as the base peak at *m/z* 383.3, while $[2M+Na]^+$ is found at *m/z* 743.5.

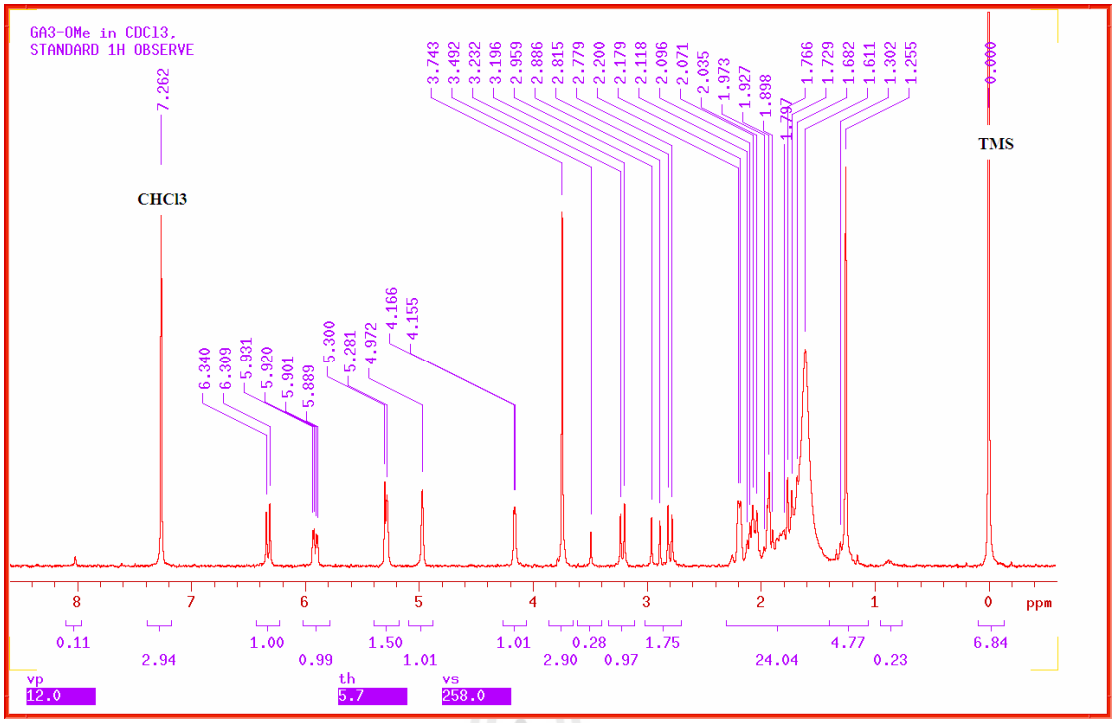



**Figure 3.11** The $^{1}$H NMR spectrum of $GA_3$-OMe in $CDCl_3$.

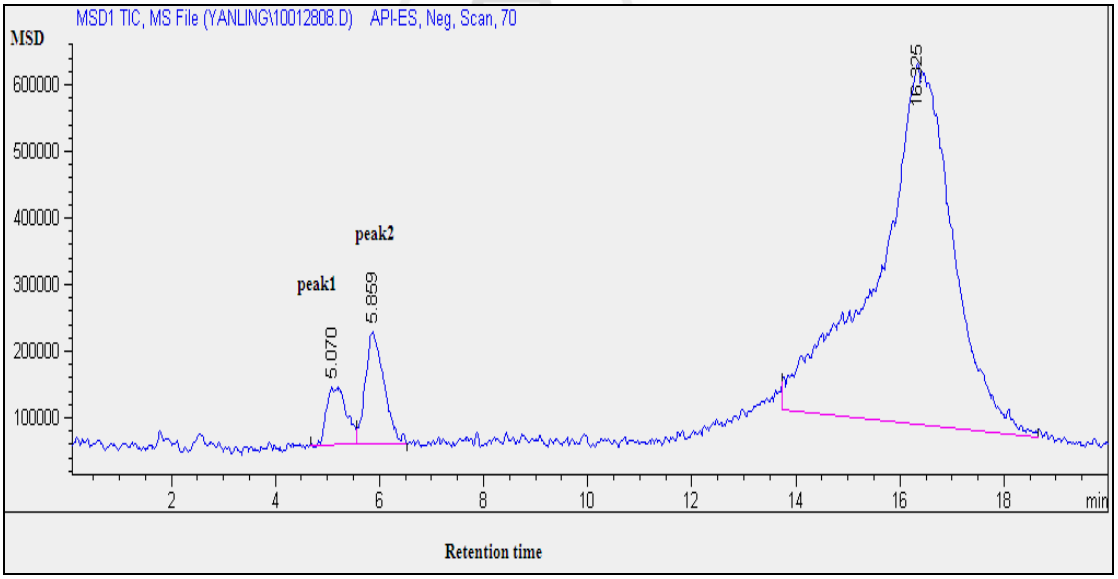
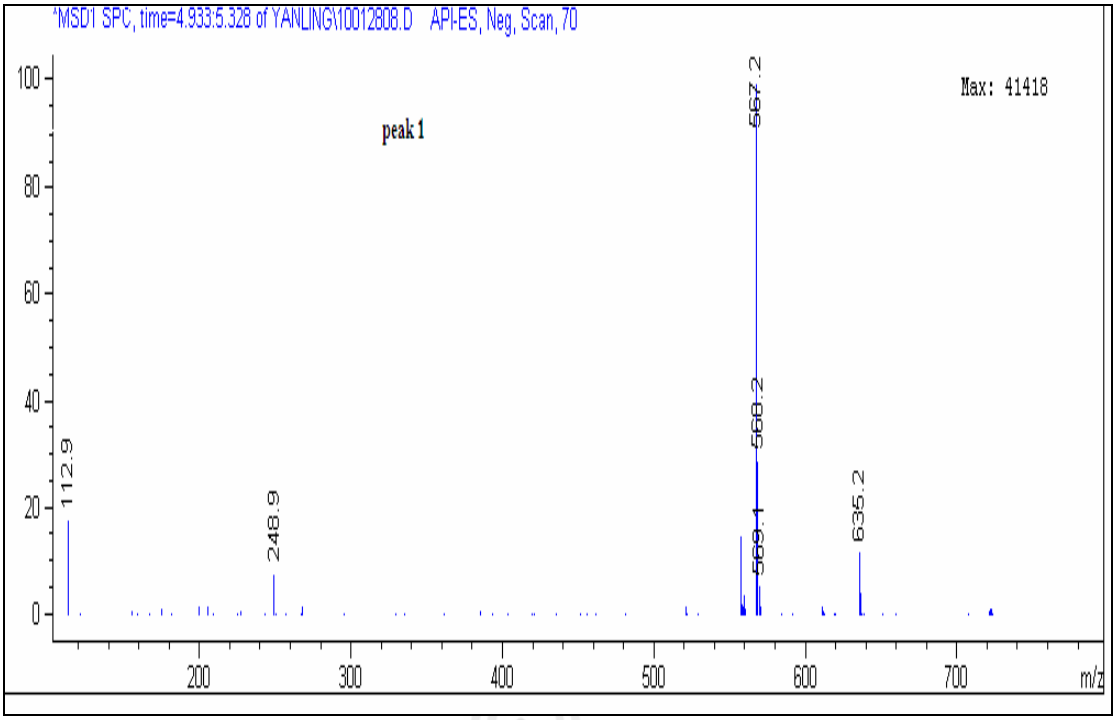



**Figure 3.12** The LC-MS chromatogram of the $GA_3$-OMe-glucosides. The compounds were separated on a ZORBAX Eclipse XDB-C18, 4.6*150 mm, 5 Micron column (Aglient). Peak 1: $GA_3$-OMe-13-O-β-D-Glc; Peak 2: $GA_3$-OMe-3-O-β-D-Glc.



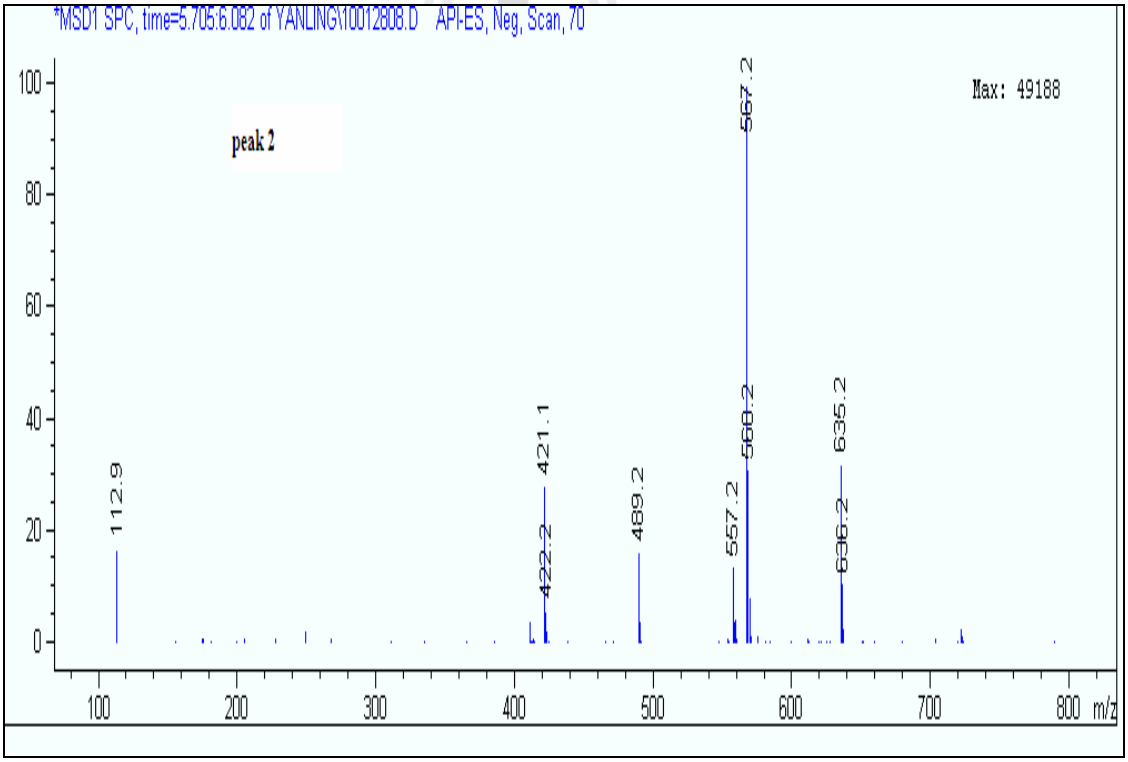




**Figure 3.13** The mass spectra of the two $GA_3$-OMe-glucosides in the negative mode. The peak at *m/z* 567.2 was assigned as $[M+HCO_2]^-$.

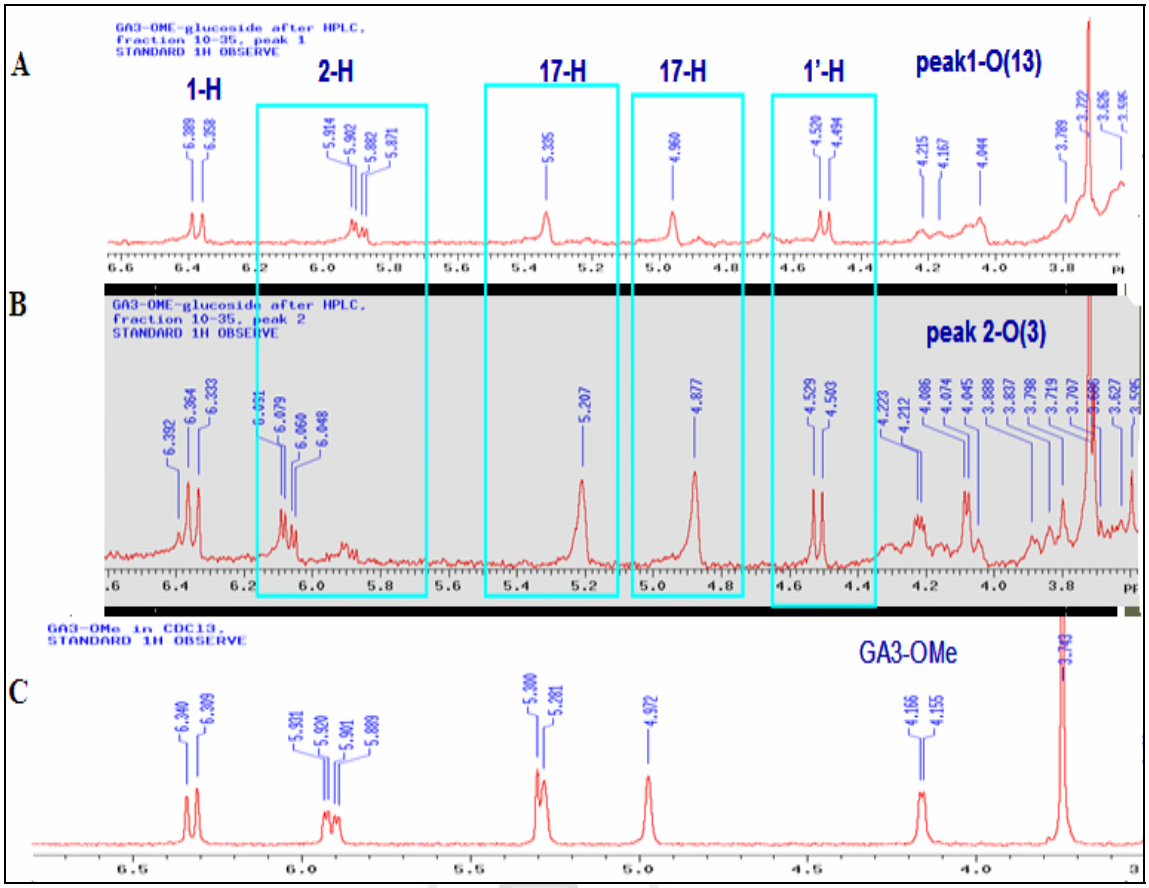



**Figure 3.14** Overlay of the NMR spectra of $GA_3$-OMe and its glucosides. A, peak 1, $GA_3$-OMe-13-O-Glc; B, peak 2, $GA_3$-OMe-3-O-Glc; C, $GA_3$-OMe.

### 3.1.4 Synthesis of gibberellin $GA_4$ methyl ester and its glucosides

The structures of $GA_4$-OMe and $GA_4$-OMe-Glc were mainly confirmed by their mass spectra (Figures 3.15 and 3.16). The peaks for the $GA_4$-OMe and $GA_4$-OMe-Glc formate adducts $[M+HCO_2]^-$ were identified as the base peaks in their negative mode mass spectra, at *m/z* 391.1 and 553.3, respectively.

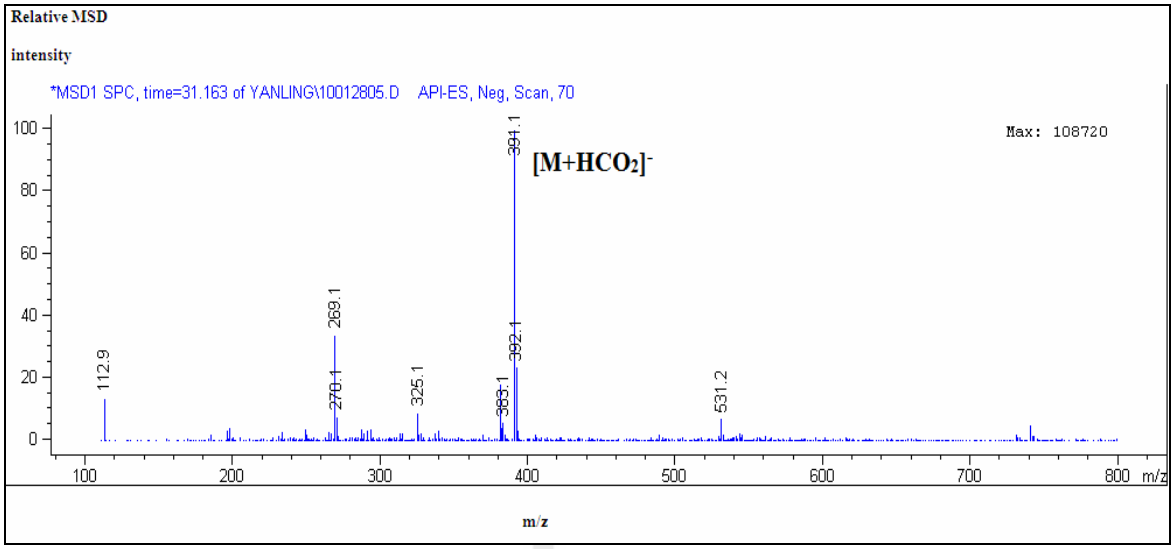



**Figure 3.15** The mass spectrum of $GA_4$-OMe in the negative mode. The peak at *m/z* 391.1 was assigned as $[M+HCO_2]^-$ .

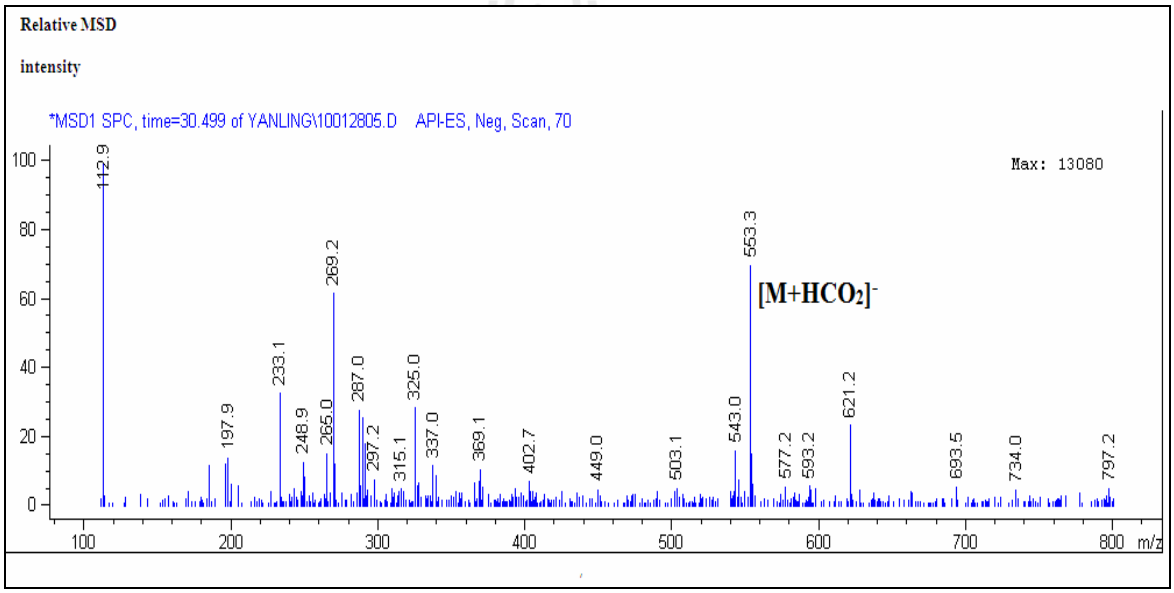



**Figure 3.16** The mass spectrum of $GA_4$-OMe-Glc in the negative mode. The peak at *m/z* 553.3 was assigned as $[M+HCO_2]^-$ .

## 3.2 Extraction, Purification and Characterization of $GA_4$-Glucosyl Ester β-Glucosidase from Rice

### 3.2.1 Screening of β-glucosidases from different rice tissues

The proteins were extracted from whole rice seeds, glumes, 7-day rice seedlings and roots, and then partially purified by ammonium sulfate precipitation and ion exchange chromatography. The

β-glucosidase activities were found in the crude extracts and fractions from the ion exchange columns. The root extract was found to have the highest specific activity to *p*NP-glucoside, but the glume extract had the highest specific activity to the $GA_4$-GE (Figure 3.17, Table 1).

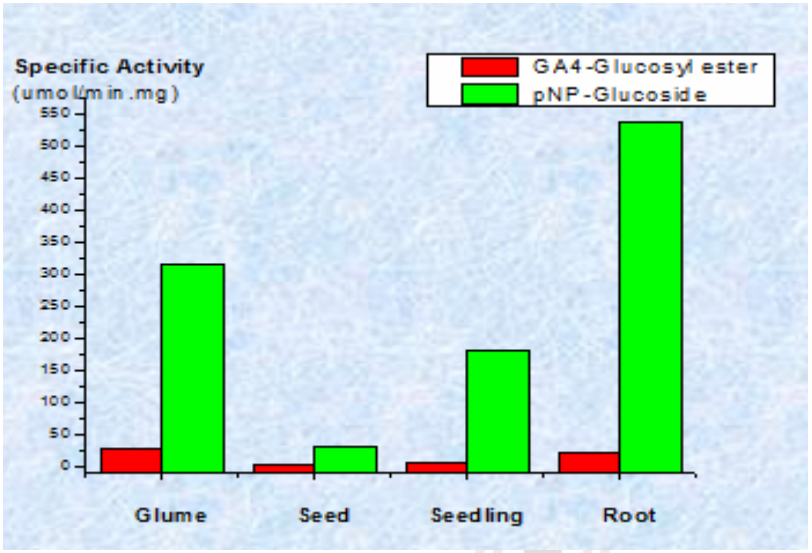



**Figure 3.17** The specific activities of different rice tissue extracts.

**Table 1.** The relative activity of β-glucosidase from different rice tissue extracts.

| | *p*NP-Glucoside | | $GA_4$-GE | |
|---|---|---|---|---|
| Tissues | Specific Activity (μmol/min.mg) | Relative Activity (%) | Specific Activity (μmol/min.mg) | Relative Activity (%) |
| Glume | 315.0 | 100 | 26.9 | 100 |
| Seed | 31.4 | 9.97 | 0.22 | 0.82 |
| Seedling | 179.7 | 57.1 | 5.11 | 19.0 |
| Root | 537.1 | 170.5 | 19.8 | 73.6 |

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

The crude protein after $(NH_4)_2SO_4$ precipitation & dialysis with McIlvaine buffer was fractionated with a ConA-Sepharose column (10 ml). Eluate showed higher activities than flow-through and crude for hydrolyzing *p*NPGlc (Figure 3.18 and Table 2).

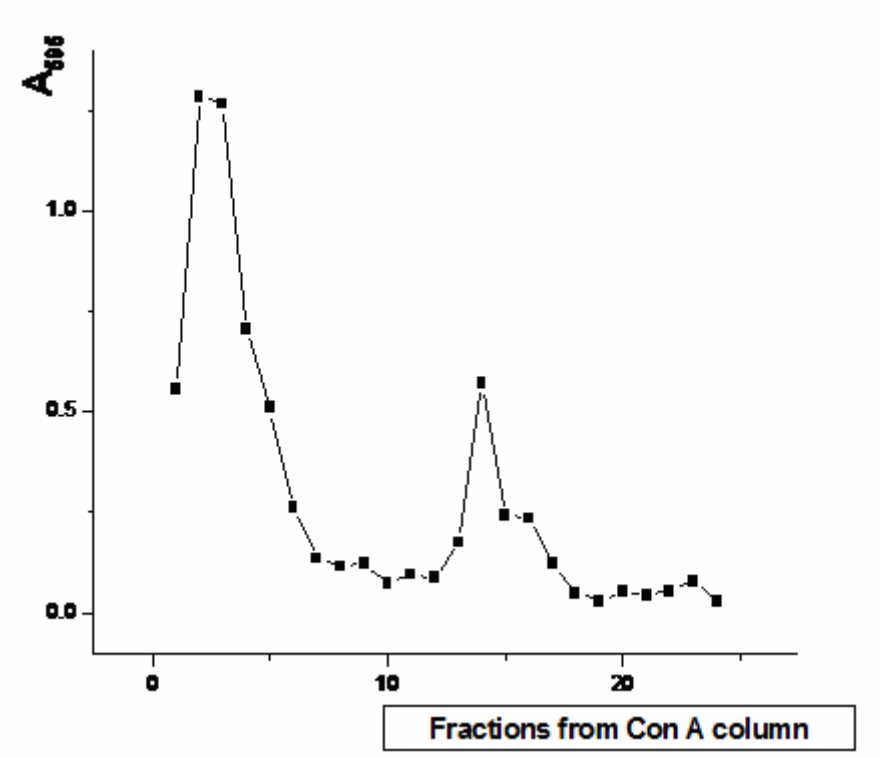



**Figure 3.18** Con A-Sepharose column elution profile of glumes extract. $A_{595}$ was the absorbance of protein at 595 nm determined with a Bio-Rad Bradford assay with brovine serum albumin (BSA) as a standard.

**Table 2.** The specific activities of fractions before and after ConA column.

| Fractions | Total protein (mg) | Specific Activity *p*NP-Glucoside (μmol/min.mg) | Purification Fold |
|---|---|---|---|
| Extraction solution | 21,800 | $2.02*10^{-3}$ | 1 |
| Pellets, after dialysis | 3,137 | $1.69*10^{-2}$ | 8 |
| Protein after Con A column | 145 | $9.41*10^{-2}$ | 47 |

### 3.2.3 Purification of β-glucosidase from rice glumes by ion exchange chromatography with a HiTrap Q-Sepharose column

The active fractions from the ConA-Sepharose column were concentrated, dialyzed and purified with a Q-Sepharose column (5 ml), which was equilibrated with 4-fold diluted McIlvaine buffer, pH 7, and eluted with a linear gradient of 0-1.0 M NaCl in this buffer (Figure 3.19). Four peaks were detected in the bound proteins, and their activities were tested with *p*NPGlc and $GA_4$-GE as described in section II (Table 3). Peak 3 showed the highest activities to hydrolyze $GA_4$-GE. These fractions were run on SDS-PAGE to check their protein compositions (Figure 3.20), and then pooled for further purification.

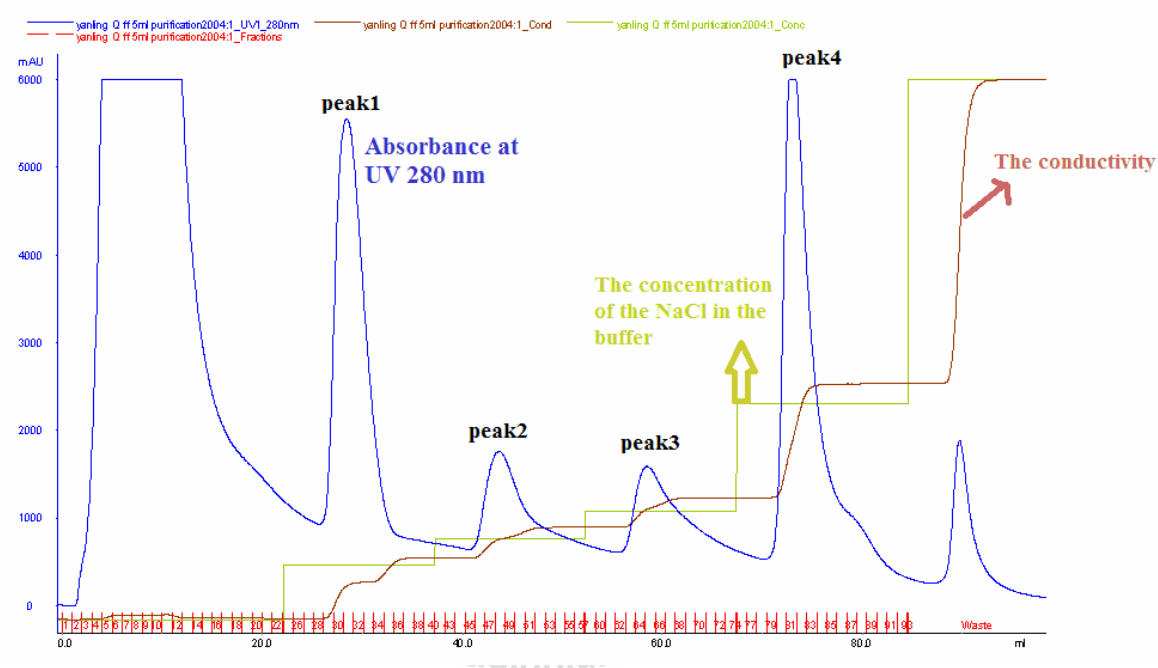



**Figure 3.19** Protein elution profile of Q sepharose ff 5 ml column. The column was eluted with a linear gradient of 0-1.0 M NaCl in 4-fold diluted McIlvaine buffer, pH 7, at a flow rate of 1.0 ml/min. The fraction volume was 1.0 ml. The blue curve indicates the absorbance at 280 nm, the green curve indicates the relative theoretical concentration of the NaCl in the buffer, and the brown curve indicates the conductivity.

**Table 3.** The specific activities of fractions from the Q sepharose FF anion exchange column.

| Fractions | Specific Activity *p*NP-Glucoside (µmol/min.mg) | Specific Activity $GA_4$-GE (µmol/min.mg) | Purification Fold (based on activity with *p*NP-Glucoside) | Relative Activity $GA_4$-GE/*p*NP-Glc (%) |
|---|---|---|---|---|
| Unbound | $7.52*10^{-2}$ | $5.25*10^{-3}$ | 37 | 7 |
| Peak1 | 0.242 | $1.17*10^{-2}$ | 120 | 4.8 |
| Peak2 | 0.265 | $1.48*10^{-2}$ | 131 | 5.6 |
| Peak3 | 0.381 | $2.44*10^{-2}$ | 189 | 6.4 |
| Peak4 | 0.157 | $1.51*10^{-2}$ | 78 | 9.6 |

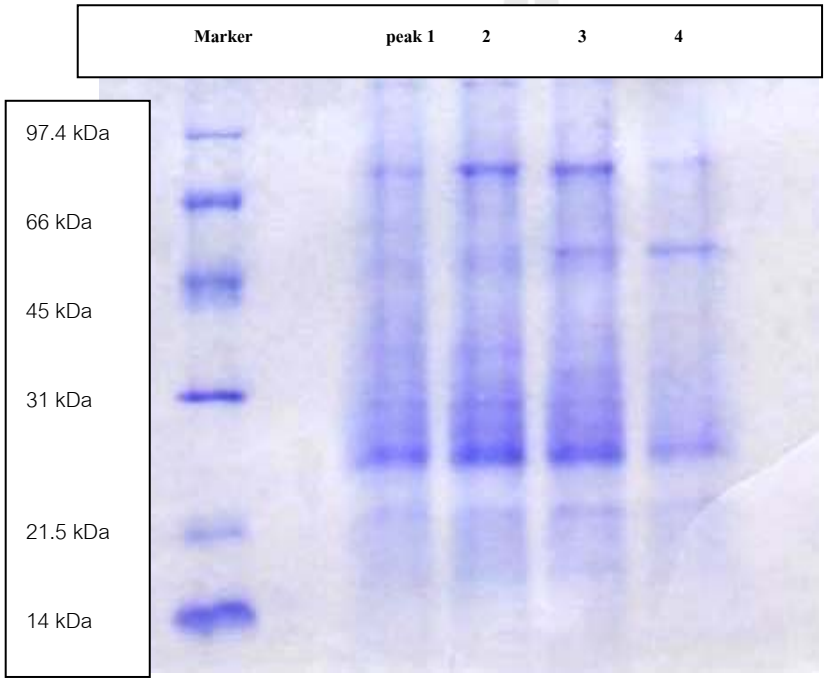



**Figure 3.20** SDS-PAGE of fractions from Q sepharose column.

### 3.2.4 Purification of β-glucosidase from rice glumes with a 2nd HiTrap Q-Sepharose column

The fractions of peak 3 and peak 4 from Q sepharose column were concentrated seperately, dialyzed and purified with a 2rd Q-Sepharose column (5 ml), which was equilibrated with 4-fold diluted McIlvaine buffer, pH 5.5. The proteins were eluted with a linear gradient of 0-1.0 M NaCl in this buffer (Figures 3.21 and 3.22). Four peaks were detected in the bound proteins, and their activities were tested with *p*NPGlc and $GA_4$-GE, as described in section 2 (Tables 4 and 5). These fractions were

run on SDS-PAGE to check their protein compositions (Figures 3.23 and 3.24), and then pooled for further purification.

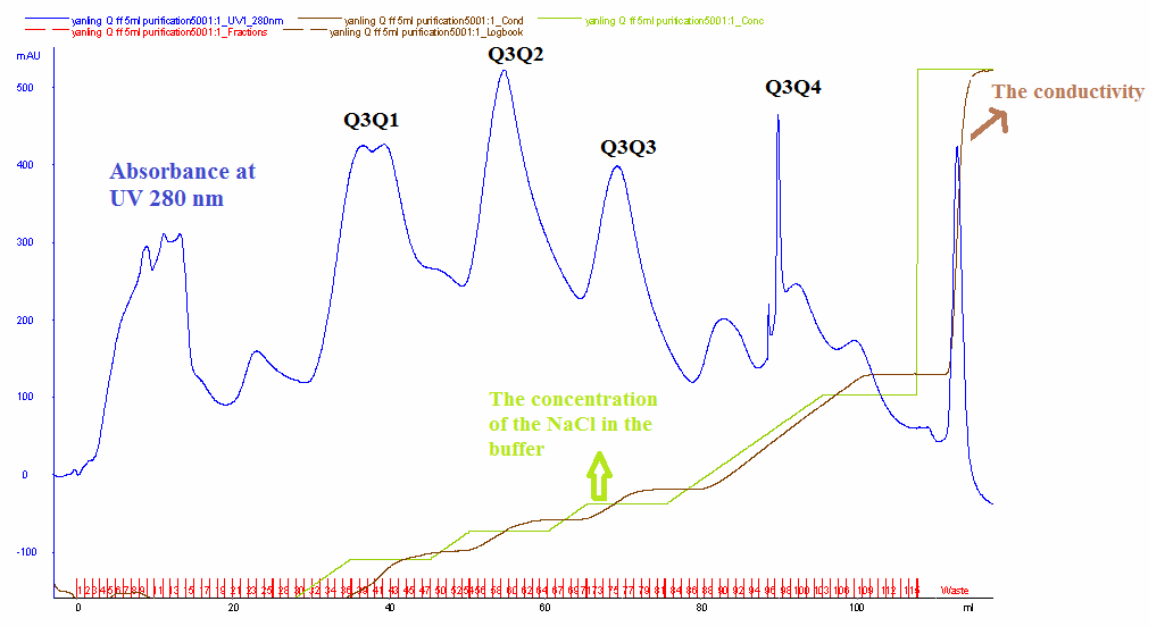



**Figure 3.21** Peak 3 elution profile by 2nd Q Sepharose column. The column was eluted with a linear gradient of 0-1.0 M NaCl in 4-fold diluted McIlvaine buffer, pH 5.5, at a flow rate of 1.0 ml/min. The fraction volume was 1.0 ml. The blue curve indicates the absorbance at 280 nm, the green curve indicates the relative theoretical concentration of the NaCl in the buffer, and the brown curve indicates the conductivity.

**Table 4.** The specific activities of fractions from 2nd Q sepharose FF column for peak 3.

| Fractions | Specific Activity *p*NP-Glucoside (µmol/min.mg) | Specific Activity $GA_4$-GE (µmol/min.mg) | Purification Fold (based on activity with *p*NP-Glucoside) | Relative Activity $GA_4$-GE/*p*NP-Glc (%) |
|---|---|---|---|---|
| Q3Q1 | $5.95*10^{-2}$ | $4.43*10^{-3}$ | 29 | 7.4 |
| Q3Q2 | 0.103 | $1.34*10^{-2}$ | 51 | 13 |
| Q3Q3 | 0.387 | $5.68*10^{-2}$ | 192 | 14.7 |
| Q3Q4 | 0.653 | $6.84*10^{-2}$ | 323 | 10.5 |

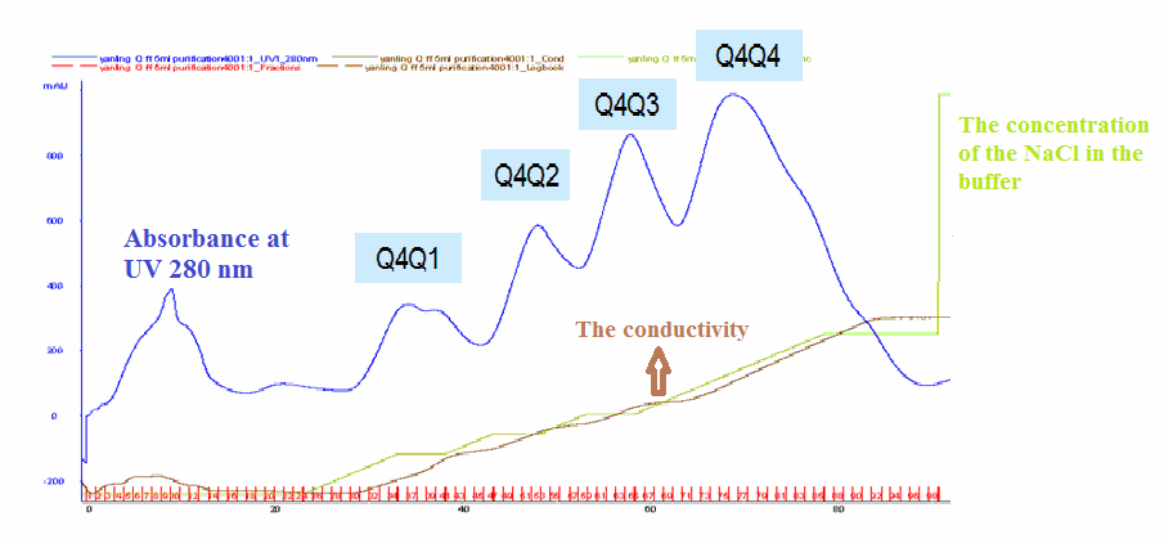



**Figure 3.22** Peak 4 elution profile by 2nd Q Sepharose column. The column was eluted with a linear gradient of 0-1.0 M NaCl in 4-fold diluted McIlvaine buffer, pH 5.5, at a flow rate of 1.0 ml/min. The fraction volume was 1.0 ml. The blue curve indicates the absorbance at 280 nm, the green curve indicates the relative theoretical concentration of the NaCl in the buffer, and the brown curve indicates the conductivity.

**Table 5.** The specific activities of peak 4 fractions from 2nd Q sepharose FF column

| Fractions | Specific Activity *p*NP-Glucoside (µmol/min.mg) | Specific Activity $GA_4$-GE (µmol/min.mg) | Purification Fold (based on activity with *p*NP-Glucoside) | Relative Activity $GA_4$-GE/*p*NP-Glu (%) |
|---|---|---|---|---|
| Q4Q1 | 0.110 | $7.86*10^{-3}$ | 54 | 7.1 |
| Q4Q2 | 0.101 | $6.64*10^{-3}$ | 50 | 6.6 |
| Q4Q3 | 0.245 | $2.22*10^{-2}$ | 121 | 9.1 |
| Q4Q4 | 0.480 | $3.05*10^{-2}$ | 238 | 6.4 |

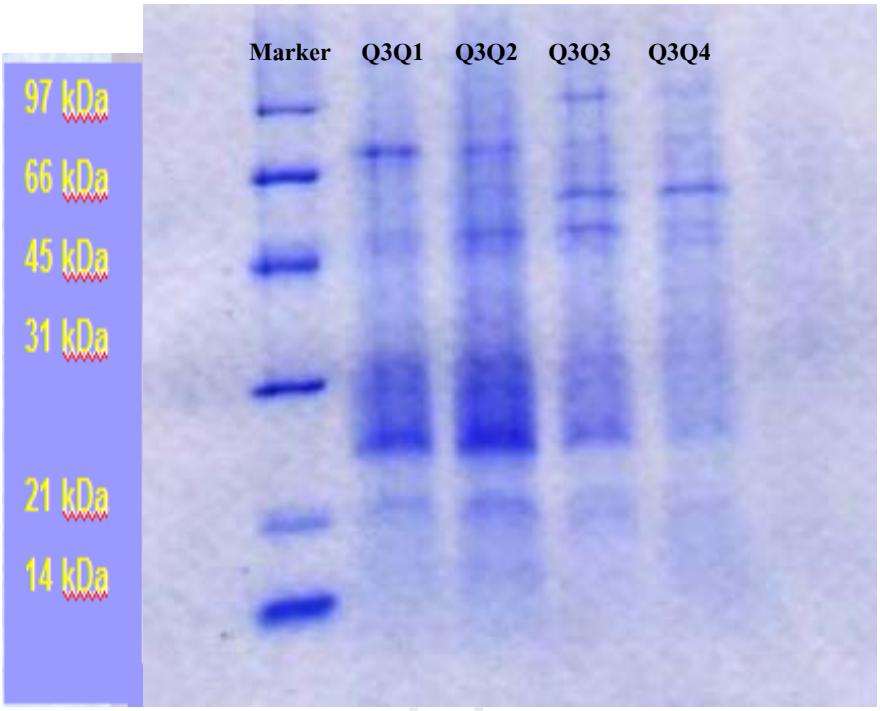



**Figure 3.23** SDS-PAGE of fractions for peak 3 from 2nd Q sepharose column.

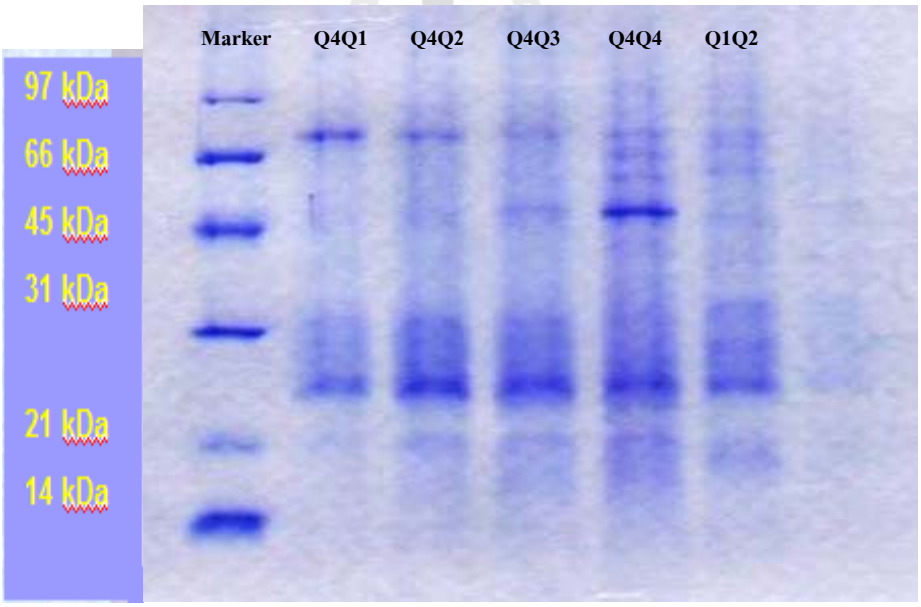



**Figure 3.24** SDS-PAGE of fractions for peak 4 from 2rd Q sepharose column.

### 3.2.5 Purification of β-glucosidase from rice with a 3rd HiTrap Q-Sepharose column

The fractions of peak Q4Q4 from Q sepharose column were concentrated, dialyzed and purified with a 3rd Q-Sepharose column (1 ml). The column was equilibrated with 4-fold diluted McIlvaine buffer, pH 5.5 and eluted with a linear gradient of 0-1.0 M NaCl in this buffer. Two peaks

were detected in the bound proteins, and their activities were tested with *p*NPGlc and $GA_4$-GE as described in section II (Table 6). These fractions were run on SDS-PAGE to check their protein compositions (Figure 3.25).

**Table 6.** The specific activities of peak Q4Q4's fractions from Q sepharose FF 1 ml column.

| Fractions | Specific Activity *p*NP-Glucoside (µmol/min.mg) | Specific Activity $GA_4$-GE ester (µmol/min.mg) | Purification Fold (based on activity with *p*NP-Glucoside) | Relative Activity $GA_4$-GE/*p*NP-Glc (%) |
|---|---|---|---|---|
| Q4Q4 | 0.480 | $3.05*10^{-2}$ | 238 | 6.4 |
| Q4Q4Q3 | 1.28 | $7.95*10^{-2}$ | 634 | 6.2 |
| Q4Q4Q4 | 1.63 | $8.7*10^{-2}$ | 807 | 5.3 |

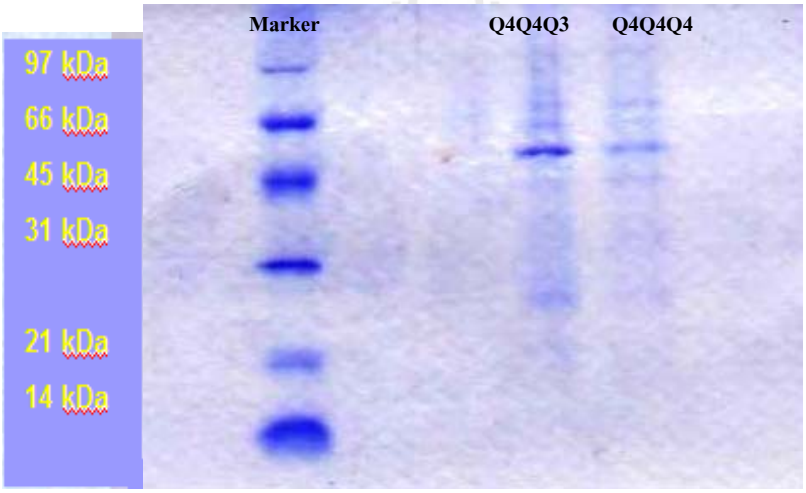



**Figure 3.25** SDS-PAGE of fractions from Q sepharose FF 1 ml column

### 3.2.6 Identification of β-glucosidase with LC-MS

The protein bands from Q sepharose column fraction were identified by LC-MS of tryptic peptides generated from their SDS-PAGE gel bands, as described in section 2.2.2.6. The peptide mass results were used in a MASCOT search of the Genbank *non-redundant (*nr) protein database (Table 7 and 8). No β-glucosidases were matched.

**Table 7. MASCOT** search results for protein band Q4Q1.

| Name | Score | % Probability | Peptide Matches | Coverage | Mw | pI | Description |
|---|---|---|---|---|---|---|---|
| 1. 125577329 | 11.9257 | 17.708794 | 1 | 1.1294 | 109082.1641 | 5.9546 | hypothetical protein OsJ 032760 Oryza sativa japonica cultivar group |
| 2. 125534579 | 11.9216 | 17.63634 | 1 | 1.127 | 109225.1797 | 5.8337 | hypothetical protein OsI 035086 Oryza sativa indica cultivar group |
| 3. 108864437 | 11.8374 | 16.21216 | **1** | 1.0806 | 113927.5078 | 5.85 | Glycosyl hydrolases family 38 protein expressed Oryza sativa japonica cultivar group |
| 4. 115485699 | 11.8334 | 16.14743 | 1 | 1.0784 | 114085.5781 | 5.85 | Os11g0525600 Oryza sativa japonica cultivar group |
| 5. 113645215 | 11.8334 | 16.14743 | 1 | 1.0784 | 114085.5781 | 5.85 | Os11g0525600 Oryza sativa japonica cultivar group |
| 6. 77551210 | 11.8334 | 16.14743 | 1 | 1.0784 | 114085.5781 | 5.85 | Glycosyl hydrolases family 38 protein expressed Oryza sativa japonica cultivar |

**Table 8. MASCOT** search results for protein band Q4Q4Q3.

| Name | Score | % Probability | Peptide Matches | Coverage | Mw | pI | Description |
|---|---|---|---|---|---|---|---|
| 1. 115435074 | 11.7978 | 15.582698 | 2 | 4.1754 | 52165.3867 | 4.9369 | Os01g0196600 Oryza sativa japonica cultivar group |
| 2. 14209591 | 11.7978 | 15.582698 | 2 | 4.1754 | 52165.3867 | 4.9369 | putative nucleotide diphosphatase Oryza sativa Japonica Group |
| 3. 125569379 | 11.7978 | 15.582698 | 2 | 4.1754 | 52165.3867 | 4.9369 | hypothetical protein OsJ 000719 Oryza sativa japonica cultivar group |
| 4. 113531826 | 11.7978 | 15.582698 | 2 | 4.1754 | 52165.3867 | 4.9369 | Os01g0196600 Oryza sativa japonica cultivar group |
| 5. AF245483_1 | 11.7978 | 15.582698 | 2 | 4.1754 | 52165.3867 | 4.9369 | OSE4 Oryza sativa |
| 6. 818849 | 11.7978 | 15.582698 | 2 | 4.1754 | 52165.3867 | 4.9369 | nucleotide pyrophosphatase precursor |
| 7. 125524775 | 10.9245 | 6.50689 | 2 | 3.3557 | 65496.9453 | 4.9552 | hypothetical protein OsI 000736 Oryza sativa indica cultivar group |

### 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 2.2.2.7. As shown in Table 9, 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 9.** $GA_4$-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].



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# 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. $GA_3$-OMe-3-O-Glc, $GA_3$-OMe-13-O-Glc and $GA_4$-OMe-Glc were synthesized with 3 steps; the final yields were less than 5% for all of them. $GA_3$-OMe-3-O-Glc and $GA_3$-OMe-13-O-Glc could not be separated by flash column chromatography, so they were separated by HPLC with multiple injections and manual collection, which was very time consuming. Furthermore, $GA_3$-OMe-3-O-Glc, $GA_3$-OMe-13-O-Glc and $GA_4$-OMe-Glc should be taken through one more reduction step to reduce -OMe to -OH, in order to obtain the natural substrates $GA_3$-3-O-Glc, $GA_3$-13-O-Glc and $GA_4$-Glc glucosides (Figure 1.5), and this reduction step is usually low yield. Therefore, it may be useful to explore an alternative synthesis method in the future.

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, 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, I 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

The proteins which could hydrolyze $GA_4$-GE to $GA_4$ were found in the crude extracts of glumes, seeds, seedlings and roots, as well as their fractions from the ion exchange columns; but the pure protein with high activity has yet to be separated. The low concentration of β-glucosidase in the rice tissues maybe a main cause. Further purification work will be increasing scale of extraction, producing more proteins in order to purify to get pure proteins and characterize their primary structures.

### 4.1.3 Screening of rice GH1 enzymes for $GA_4$-GE hydrolysis

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 9). 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. Therefore, Os3BGlu6 hydrolyzes $GA_4$-GE better and with a higher preference than Os4BGlu12 and other rice GH1 β-D-glucosidases, such as Os3BGlu7.

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 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, it serves as a good model for investigation of β-glucosidic hydrolysis of glucosyl esters.

## 4.2. Conclusions and Comments

Nine GA conjugates, which included acetylated and deacetylated $GA_4$-GE, acetylated and deacetylated $GA_3$-GE, $GA_4$-OMe, $GA_3$-OMe, $GA_3$-OMe-3-O-Glc, $GA_3$-OMe-13-O-Glc and $GA_4$-OMe-Glc were synthesized following the published methods or with modification. Their structures were identified with $^1$H 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.

The β-glucosidase activities were found in the crude extracts of glumes, seeds, seedlings and roots, as well as their fractions from the ion exchange columns; but the pure protein with high activity has not been separated yet.

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.

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   Instrumental analysis on GC, GC-MS/MS, NMR, FT-IR, HPLC, LC-MS, FPLC; Multi-step synthesis and structure analysis of organic compounds; extraction of natural product; purification and characterization of proteins, enzyme's activity assay.

7. Research Experience

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7.1. Journal Publications

**(1)** Hua Y, Sansenya S, Saetang C, Wakuta S, Ketudat Cairns JR. (2013). Enzymatic and structural characterization of hydrolysis of gibberellin A4 glucosyl ester by a rice β-D-glucosidase. Arch. Biochem. Biophys. 537(1): 39-48.

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

**(3)** 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.

**(4)** 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.

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**(7)** Chen SS, Hua YL, Lei ZP. (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/หรือโครงการวิจัย แหล่งทุน และสถานภาพในการทำวิจัยว่าได้ทำการวิจัยลุล่วงแล้วประมาณร้อยละเท่าใด)

None

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   7.1 Journal Publications

7.1.1. Cairns JR, Williamson MK, Price PA. 1991. Direct identification of β-carboxyglutamic acid in the sequencing of vitamin K-dependent proteins. *Analytical Biochemistry* **199**, 93-97.

7.1.2. Cairns JR, Price PA. 1994. Direct demonstration that the vitamin K-dependent bone Gla protein is incompletely β-carboxylated in humans. *Journal of Bone and Mineral Research* **9**, 1989-1997.

7.1.3. Svasti J, Srisomsap C, Surarit R, Techasakul S, and Ketudat-Cairns JR. 1998. Characterization of a novel rotenoid-β-glucosidase and its natural substrate from Thai rosewood. *Journal of Pure and Applied Chemistry* **70** (11).

7.1.4. Ketudat Cairns JR, Chantarangsee M, Chaiwangrad S, Phawong J. 1999. Primary structure-based screening for Glycosyl hydrolases in Thai plants. *Thai Journal of Biotechnology* **1**, 20-30.

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

7.1.11. 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.

7.1.12. Ketudat Cairns JR, Keeratichamroen S, Sukcharoen S, Champattanachai V, Ngiwsara L, Lirdprapamongkol K, Liammongkolkul S, Srisomsap C, Surarit R, Wasant P and Svasti J. 2005. The molecular basis of mucopolysaccharidosis Type 1 in two Thai patients. *Southeast Asian Journal of Tropical Medicine and Public Health* **36**, 1308-1312.

7.1.13. Toonkool P, Metheenukul P, Sujiwattanarat P, Paiboon P, Tongtubtim N, Ketudat-Cairns M, Ketudat-Cairns J, Svasti J. 2006. Expression and purification of dalcochinase, a β-glucosidase from *Dalbergia cochinchinensis* Pierre, in yeast and bacterial hosts. *Protein Expression and Purification* **48**, 195-204.

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

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

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

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

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

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

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

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

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

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

7.2.10. 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, to be completed 2556/09.

7.3. Completed Research Projects (งานวิจัยที่ทำเสร็จแล้ว : ชื่อแผนงานวิจัย และ/หรือ โครงการวิจัย ปีที่พิมพ์ การเผยแพร่ และสถานภาพในการทำวิจัย) See Sections 7.1 and 7.2. (7.2.1-7.2.10), No project sets organized have been completed.

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

7.4.1. Structure, function and application of plant β-glucosidases and related enzymes, 2556-2559, Thailand Research Fund Basic Research Grant BRG53800. Head of project.

7.4.2. Identification and characterization of rice gibberellin beta-glucosidase. 2554-2557, SUT, Budget Bureau, NRCT.

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