STUDY OF A NOVEL β-GLUCOSIDASE FROM THAI PLANTS

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STUDY OF A NOVEL β-GLUCOSIDASE FROM THAI PLANTS

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ABSTRACT

β-glucosidases (EC 3.2.1.21) are a group of glycosidase enzymes (EC 3.2) that catalyze the hydrolysis of β-glucosidic linkages formed between D-glucose and the hydroxyl group of other compounds (aglycone or another glycone). These enzymes have potential applications for the sequence determination of oligosaccharides, as well as for oligosaccharide or glycoside synthesis by reverse hydrolysis or transglucosylation. We have used these enzymes (Thai rosewood, almond or cassava stem β-glucosidase) to screen for natural β-glucosides in 15 plant species. The ethanol extract of plant tissues were incubated in the presence or absence of enzyme, and the products separated by TLC, followed by detection under UV or spraying with 10% H_2SO_4 in absolute ethanol. The results showed that β-glucosides were found in many plants especially in *Plumeria obtusa* Linn, *Plumeria rubra* Linn and *Murraya paniculata* Jack. In addition, β-glucosidases were also screened for by using its crude substrate and other substrates e.g. arbutin and pNP-Glc. The results show that the crude enzyme from *Plumeria obtusa* Linn contains hydrolytic activity to its crude substrate and pNP-Glc. Subsequently, a β-glucosidase was purified from the flowers *Plumeria obtusa Linn* by DEAE-Cellulose, Con A-Sepharose, Sephacryl S-300 and Butyl-Sepharose chromatography. The cumulative yield and fold purification of the β-glucosidase were 2.3 % and 143 fold, respectively. The enzyme has a molecular weight of 180,000 in the native state, and shows 2 bands of 59,000 kDa and 67,000 kDa in the denatured state. One fluorescent band of β-glucosidase was found with pI about 5.0 by agarose gel electrophoresis. The optimum pH of the purified β-glucosidase detected by pNP-Glc and its natural substrate was 5.5. The K_m value for pNP-Glc and *Plumeria* β-glucoside were 2.9 and 0.33 mM, respectively. The enzyme had hydrolytic activity towards pNP-β-Glc, pNP-β-Fuc, pNP-β-Gal and pNP-β-Man, and had lower activity for other pNP-glycosides, dalcochinin-β-glucoside (the natural substrate of Thai Rosewood β-glucosidase) and esculin, The enzyme could not hydrolyze cyanogenic glucoside (prunasin, linamarin and amygdalin), the natural substrate of *Solanum torvum* (torvoside H), disaccharides (gentiobiose), aromatic glucosides (arbutin and salicin) and alkyl glucosides (methyl-glucosides and hexyl-glucosides). The natural substrate of β-glucosidase from the flowers of *Plumeria obtusa* Linn was isolated and identified. Structure elucidation of the compound was established by NMR spectroscopic data. It is a known iridoid glucoside, namely plumieride coumarate glucoside.

KEY WORDS : *PLUMERIA OBTUSA* LINN/ β-GLUCOSIDASE / β- GLUCOSIDE

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การศึกษาเอ็นไซม์เบต้ากลูโคซิเดสชนิดใหม่จากพืชในประเทศไทย
(STUDY OF A NOVEL β-GLUCOSIDASE FROM THAI PLANTS)

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บทคัดย่อ

เบต้ากลูโคซิเดสเป็นเอ็นไซม์ที่ย่อยพันธะเบต้ากลูโคซิดิคระหว่างน้ำตาลกลูโคสกับน้ำตาลตัวอื่นๆหรือสารชนิดอื่น และยังสามารถใช้หาลำดับของโอลิโกแซคคาไรด์และสังเคราะห์โอลิโกแซคคาไรด์และไกลโคไซด์ด้วยปฏิกริยาไฮโดรไลสิสแบบย้อนกลับหรือปฏิกิริยาทรานกลูโคซิลเลชั่น นอกจากนี้ยังได้ใช้เอนไซม์ชนิดนี้ในการตรวจหาเบต้ากลูโคไซด์ในพืช 15 ชนิดโดยใช้เอนไซม์สามชนิด คือ เบต้ากลูโคซิเดสจากพยูง อัลมอนด์ และต้นมันสำปะหลัง พบว่าในลั่นทมขาว ลั่นทมแดงและแก้วมีปริมาณกลูโคไซด์ที่สามารถตรวจพบได้ นอกจากนี้ยังได้มีการตรวจหาเอนไซม์เบต้ากลูโคซิเดสโดยใช้สับเสรทสำหรับเอนไซม์นี้ในการตรวจหา พบว่าสารสกัดจากดอกลั่นทมขาวสามารถย่อยสับสเตรทของตัวมันเองและ pNP-Glc ได้ด้วย แสดงให้เห็นว่ามีเอนไซม์เบต้ากลูโคซิเดสและเบต้ากลูโคไซด์อยู่ในดอกลั่นทมขาว จากนั้นจึงทำการสกัดเอนไซม์เบต้ากลูโคซิเดสจากดอกลั่นทมขาวโดยวิธีทางโครมาโตกราฟีโดยใช้ DEAE-Cellulose, Con A-Sepharose, Sephacryl S-300 และ Butyl-Sepharose คอลัมน์ จากขั้นตอนที่กล่าวมาพบว่า มีปริมาณแอคติวิตี้ที่หลงเหลือเท่ากับ 2.3 เปอร์เซ็นต์ และมีความบริสุทธิ์เพิ่มขึ้น 143 เท่า ขนาดโมเลกุลของเอ็นไซม์ที่ได้จากคอลัมน์เซฟาคริล เอส-300 คือ 180 กิโลดาลตัน และพบว่ามีขนาด 59 และ 67 กิโลดาลตันบนอิเลคโตรโฟรีซีส เอ็นไซม์นี้มีค่า pI ประมาณ 5.0 และมีค่า pH ที่ให้แอคติวิตี้ต่อ pNP-Glc และสับสเตรทธรรมชาติสูงสุด คือ 5.5 จากการศึกษาทางด้านจลศาสตร์ของเอ็นไซม์ต่อ pNP-Glc และ สับสเตรทธรรมชาติได้ค่า K_m เท่ากับ 2.9 และ 0.33 มิลลิโมลาร์ตามลำดับ เอ็นไซม์นี้สามารถย่อย pNP-β-Glc, pNP-β-Fuc, pNP-β-Gal และ pNP-β-Man ในขณะที่ย่อย pNP-glycosides อื่นๆ ได้น้อยกว่า นอกจากนี้เอ็นไซม์ยังสามารถย่อย dalcochinin-β-glucoside และ esculin ได้ สับสเตรทธรรมชาติของเบต้ากลูโคซิเดสจากดอกลั่นทมขาวได้ถูกแยกและศึกษาโครงสร้างโดยเทคนิคทาง NMR พบว่าเป็นกลูโคไซด์ที่จัดอยู่ในพวก iridoid ที่มีชื่อว่า plumieride coumarate glucoside

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LIST OF ABBREVIATIONS

A	absorbance
$AmSO_4$	ammonium sulfate
α-	alpha-
β-	beta-
δ	chemical shift (in ppm)
BSA	bovine serum albumin
^oC	degree Celsius
COSY	correlated spectroscopy
d	doublets
dd	doublet of doublets
D_2O	deuterium oxide
DEAE	diethylaminoethyl
DEPT	distortionless enhancement by polarization transfer
g	gram
h	hour
HCN	hydrogen cyanide
HMBC	heteronuclear multiple bond correlation
HMQC	heteronuclear multiple quantum coherence
Hz	Hertz
kDa	kiloDalton
kg	kilogram
MeOH	methanol
M	molar
min	minutes
mm	millimetre
mM	millimolar
mU	milliunit (of enzyme activity)
μM	micromolar

LIST OF ABBREVIATIONS (CONTINUED)

MW	molecular weight
NaCl	sodium chloride
nM	nanomolar
NMR	nuclear magnetic resonance
pNP	p-nitrophenol
pNP-Fuc	p-nitrophenyl-β-D-fucopyranoside
pNP-Glc	p-nitrophenyl-β-D-glucopyranoside
pNP-Gal	p-nitrophenyl-β-D-galactopyranoside
pNP-Man	p-nitrophenyl-β-D-mannopyranoside
Rf	relative mobility
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
Spp	species
TLC	thin-layer chromatography
UV	ultraviolet
v/v	volume/volume
w/v	weight/volume

CHAPTER I
INTRODUCTION

1.1 β-Glucosidases

1.1.1 Overview of β-Glucosidases

β-glucosidases (EC 3.2.1.21) are a group of glycosidase enzymes (EC 3.2) that catalyze the hydrolysis of β-glucosidic linkages formed between D-glucose and the hydroxyl group of a compound (aglycone or other glycone). β-glucosidases are widely distributed in plants, fungi, animal and bacteria, and play an important role in a variety of fundamental biological process such as growth and development, host-parasite interaction, chemical defense and lignification. In higher plants, β- glucosidases have been implicated in such fundamental processes as chemical defense against herbivores and pathogens (1), lignification (2), and regulation of the biological activity of phytohormones by hydrolysis of their inactive hormone-glucoside conjugates (3). Almost all β-glucosidases have subunit molecular weight of 55 to 65 kD, acidic pH optima (pH 5-6), and requirement for a β-glucoside as substrate. β-glucosidases from widely different sources show a remarkable similarity in substrate specificity glycone (glucose) and some non-physiological glucosides (e.g., nitrophenol and umbelliferone). But they may have widely different physiological glucoside substrates with different aglycone moieties. In general, β-glucosidases from different sources appear to differ in their specificity for the aglycone linked to the glucosyl group by a β-glucosidic bond. The natural substrate may not be a single glucoside but includes a vast array of aglycone groups.

The β-glucosidases may be divided into 3 families (1, 3 and 9) out of some 90 families of glycoside hydrolase (4). Glycoside hydrolase family 1 are retaining enzymes that cleave the β-anomer of substrate to yield a β-anomer product. The β-

glucosidase family 1 enzymes have more than 170 types, such as almond β-glucosidase, dalcochinase and cassava linamarase, etc.

1.1.2 Plant β-Glucosidase

Plant β-glucosidases have been known for over 150 years since the description of the action of emulsin (almond β-glucosidase) on amygdalin. Many heteroglucosides are considered to be physiologically inactive, while the corresponding aglycones are active. In plants, many compounds are widely encountered as β-glucosyl conjugates. These glycosides belong to various chemical families, such as benzoic or hydroxycinnamic acids, cyanogenic glucosides, steroid glycosides and flavonoids. They are generally found in large amounts and usually stored in vacuoles. β-glucosidases hydrolyze various β-glucoside substrates, releasing aglycones that may be involved in many plant biological processes.

Plant β-glucosidases are involved in a variety of important processes such as a release of physiologically active aglycones, lignin synthesis, defense mechanisms and cell wall degradation. Plant β-glucosidases also function to activate phytohormones by releasing a active phytohormone from the phytohormone conjugated form (e.g. cytokinin, gibellerin and auxin) (5-7). β-glucosidases are also involved in defense mechanisms against herbivores and pathogens by releasing toxic aglycones such as hydroxamic acid, terpenes, saponins and cyanide (8). Moreover, β-glucosidase is an important enzyme in plant cell wall degradation, catalyzing the breakdown of cellulose (9) and promoting lignin synthesis by releasing coniferyl alcohol, a substrate of lignin, from the coniferyl glucoside form (10,11).

The β-glucosidases are found in a variety of plants such as cassava (*Manihot esculenta* Crantz) and almond (*Prunus amygdalus* Batsch). Previously, Srisomsap *et al*. reported the purification of an enzyme with β-glucosidase and β-fucosidase activity from the seeds of *Dalbergia cochinchinensis* Pierre (Thai rosewood) (12).

1.1.3 Substrate specificity of plant β-Glucosidase

β-glucosidases from different sources have widely different substrate specificity with different aglycone moieties (13), while they show similarity in substrate specificity for glycone (glucose) and some non-physiological aglycones such as nitrophenols and umbelliferone. In general, β-glucosidases from different orders and kingdoms appear to differ in their specificities for the aglycone linked to the glucosyl group by a β-glucosidic bond. The natural substrate may not be single glucosides but include the vast array of aglycone groups.

However, Hosel and Conn (14) emphasized that the role of aglycone or non-carbohydrate moieties in determining the specificity of these glucosidases for their natural substrates is largely unappreciated and suggested that more attention be directed to this matter.

1.1.4 The catalytic mechanism of β-Glucosidase

β-glucosidases are enzymes that hydrolyze the glycosidic linkages of conjugated monoglycosides. This hydrolysis takes place via general acid catalysis that requires two main amino acid residues, namely glutamic acid and/or aspartic acid: a proton donor and a nucleophile/base (15). Hydrolysis occurs via two major mechanisms, retention or inversion, at the anomeric configuration. Firstly, retaining glycoside hydrolases (Figure 1) lead to a retention of the configuration at the anomeric carbon of the substrate via a double displacement mechanism. Hydrolysis of a glycosidic bond thus creates a product with the same anomeric configuration as the substrate. The catalytic machinery of these enzymes involves two catalytic carboxylates in opposite sides of the sugar plane which perform two separate chemical steps. In the first step (glycosylation), a carboxylic group provides general acid catalysis, with the leaving group departing simultaneously with a nucleophilic attack by the second carboxylate to form a glycosyl-enzyme intermediate. In the second step (deglycosylation), the first residue functions as a general base to activate the incoming nucleophile (a water molecule in the case of hydrolysis, and an alcohol in the case of transglycosylation) which hydrolyses the glycosyl-enzyme. The distance between the two carboxylates is approximately 5.5 Å. Secondly, inverting glycoside hydrolases (Figure 2) lead to an

inversion of the anomeric configuration via a single nucleophilic displacement. Hydrolysis of a β-glycosidic bond thus creates a product with the α-configuration or vice versa. The catalytic machinery of these enzymes involves two catalytic carboxylates to provide general acid-catalysed leaving group departure and general base-assistance to the nucleophilic attack by a water molecule from the opposite side of the sugar ring. The distance between the two carboxylates is less constrained than for the retaining enzymes and is in the range 6.5-9.5 Å.

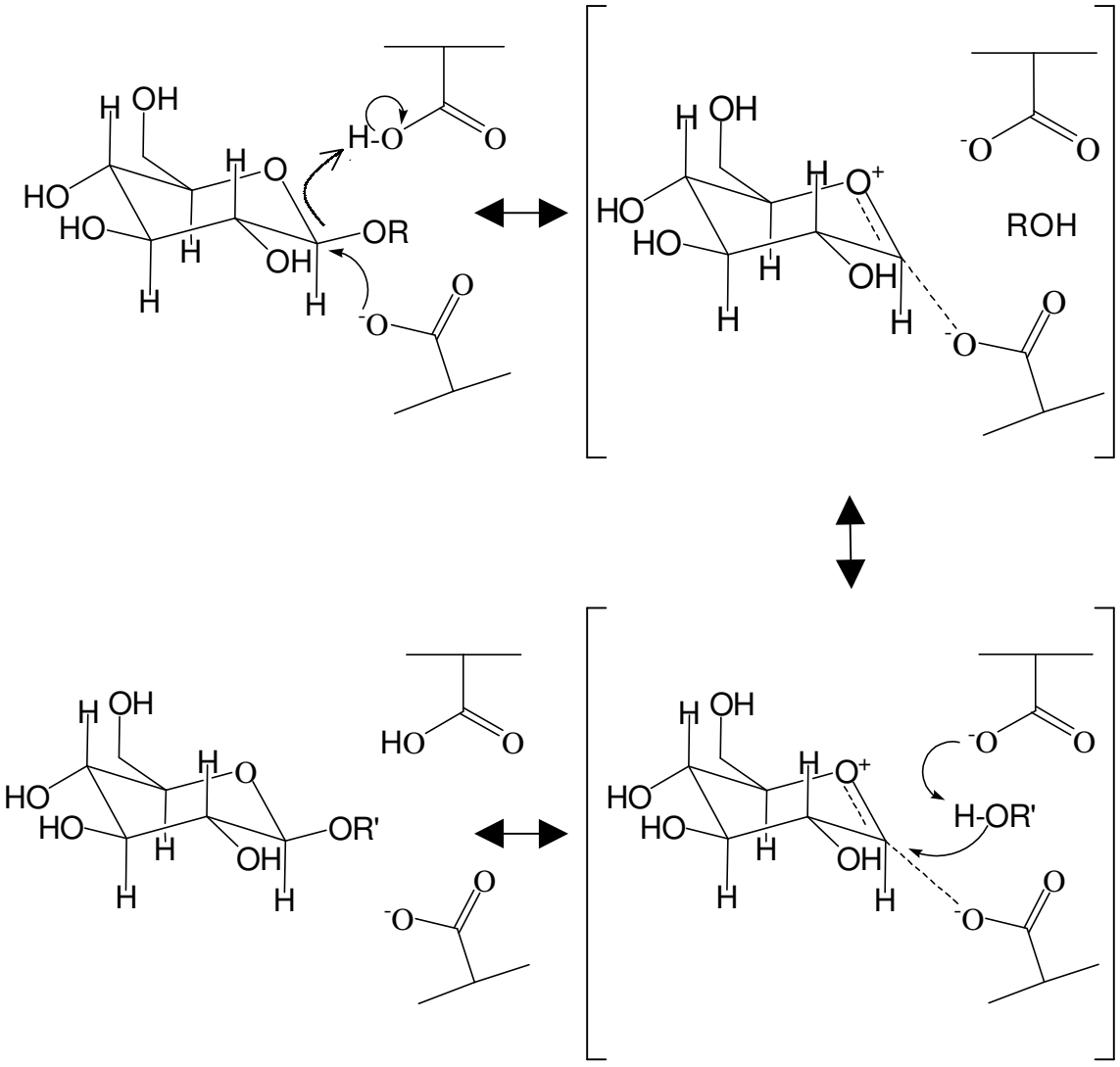


Figure 1: A mechanism of a hydrolysis reaction for a retaining β-glucosidase.

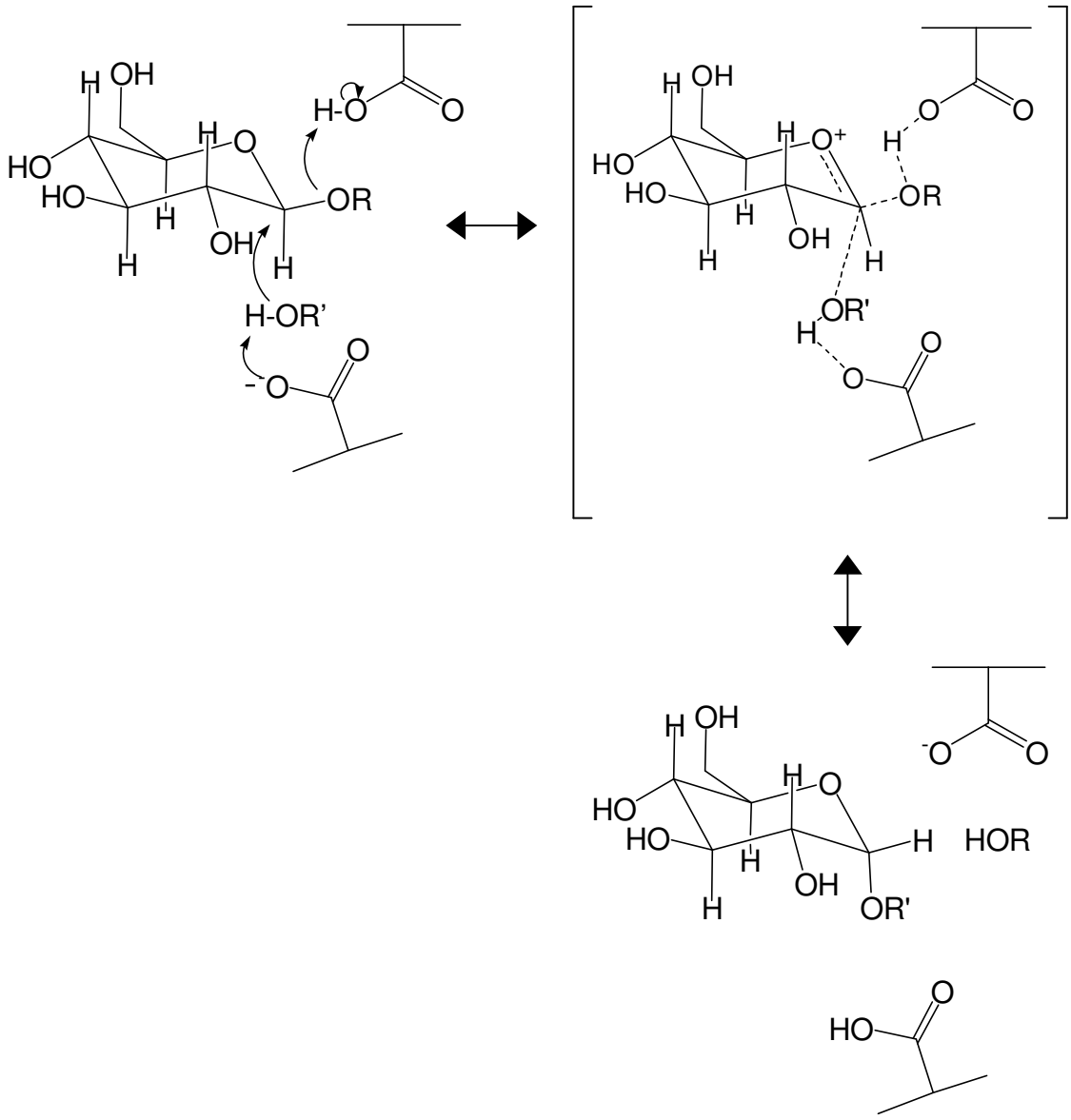


Figure 2: A mechanism of the hydrolysis reaction for an inverting β-glucosidase.

1.1.5 Applications of glycosidases

β-glycosidases are useful for sequence determination of oligosaccharides and glycans by sequential degradation. Exo-glycosidases cleave only monosaccharide residues which are located at the non-reducing terminal. They are useful in biotechnological processes such as food detoxification and beverage quality enhancement. β-glycosidases can also be used for synthesis of oligosaccharides and aryl or alkyl glucoside (16-18). Thus, it is possible to prepare various oligosaccharides that could not easily extracted from natural sources. Furthermore, β-glucosidases (as a group of glycosidase enzymes) may also be used for screening for natural β-glucosides in plants. Boonpuan *et al*. (unpublished data) reported the screening of β-glucoside in Thai plants by the incubation of ethanolic extracts of plant with Thai rosewood β-glucosidase. The reaction mixture was analyzed by TLC and detected by UV or 10% sulfuric acid in ethanol (Table 1). Preliminary results showed that the extract from *Plumeria spp.* contained a large amount of β-glucosides that could be hydrolyzed by Thai rosewood β-glucosidase. Use of the β-glucoside showed the presence of a β-glucosidase with specificity towards its natural substrate.

Table 1: Screening of β-glucosides from various Thai plants by Thai rosewood β-glucosidase

High glucoside content : (+++), medium glucoside content : (++), low glucoside content : (+) , no glucosides : (-)

Botanical name	Tissue	UV	H_2SO_4
Apocynaceae			
Plumeria acutifola Poir.	Leaves	+	+++
	Petiole	+	++
	Flower	+	+++
Plumeria alba Linn.	Leaves	+	+++
	Petiole	+	++
	Flower	+	+++
Plumeria rubra Linn.	Leaves	+	+++
	Petiole	+	++
	Flower	+	+++
Nerium inducum Mill.	Leaves	-	-
	Petiole	+	++
	Flower	+	++
Papilionacae			
Dalbergia cochinchinesis Pierre.	Seed	+++	-
Cajanus cajan Linn.	Seed	+++	+++
Rutaceae			
Murraya paniculata Jack.	Leaves	+++	+++
	Petiole	+++	+
	Flower	++	-
Acanthaceae			
Rhinacanthus nastus Kurz.	Whole plants	+++	++
Euphorbiaceae			
Jatropha gossypifolia Linn.	Rhizome	+++	++

Table 1 (continued)

Botanical name	Tissue	UV	H_2SO_4
Solanaceae			
Solanum torvum Sw.	Fruits	+++	+++
Solanum melongena Linn.	Friuts	++	+
Linociera sutepensis Kerr.	Fruits	++	-

1.2 Overview of Glycosides

A glycoside is a compound formed between a furanose or pyranose sugar and one or more nonsugar (aglycone) compounds through a glycosidic linkage (19). The glycoside structure is usually an ether-like compound, yielding O-glucosides where the linkage between the monosaccharide and the aglycone is through an oxygen, i.e., C-O-C. Less commonly, C-glycosides have a C-C linkage between monosaccharide and the aglycone. When the nucleophilic atom is nitrogen or sulphur, N-glycosides and S-glycosides are obtained with the monosaccharide aglycone linkages designated as C-N-C or C-S-C, respectively. While the monosaccharide-aglycone linkage of the O-, N- and S- is susceptible to acid, alkali or enzyme-catalyzed hydrolysis, the C-C linkage of the C-glycosides is not (20).

The sugar component of a glycoside is often D-glucose, although D- and L-galactose, D-manose, D-fructose and L-rhamnose have also been found. Some pentoses, for example, D- and L-arabinose and D-ribose, have also been detected. Some deoxy- and O-methyl sugars occur only in the cardiac glycosides. Occasionally, the carbohydrate is present as a disaccharide or in some cases as a trisaccharide. Almost all natural glycosides examined so far have the β-configuration at the anomeric center (21).

1.2.1 Natural glycosides

Natural glycosides were known long before the preparation of the alkyl glycosides by Fisher (22). The classical work of Liebig and Wohler on the oil of bitter almonds (1837) showed the presence of the crystalline glycoside amygdalin (23).

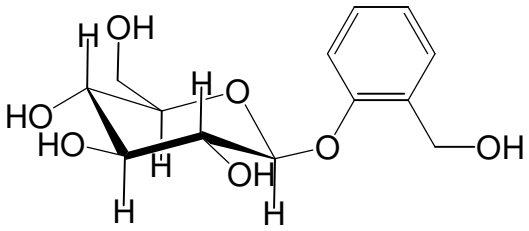
In general, there is an appropriate enzyme or a mixture of enzymes accompanying the glycosides of plants, capable of hydrolyzing the glycoside. The enzyme is naturally not contained in the same particular stage or cellular compartment of the plant.

Plant glycosides are found in leaves and seeds of plants, and in the bark of trees, and are exceedingly numerous. In the animal world also, glycosides are found as cerebrosides in nerve and brain tissue and in the nucleoproteins, containing nitrogen glycosides.

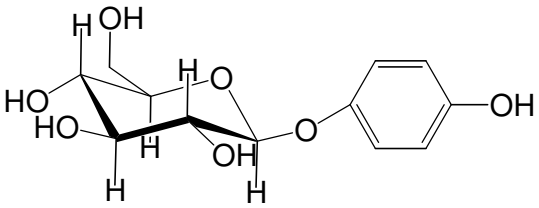
The glycosides are often present as a largely water-soluted in the vacuole, away from the essent metabolic activity of the cytoplasm. The sugar molecules make the aglycone water-soluble, so that it can move around the plant more readily. If the sugar was not attached, the aglycone could not dissolve in the vacuolar sap.

1.2.2 Function of natural glycosides

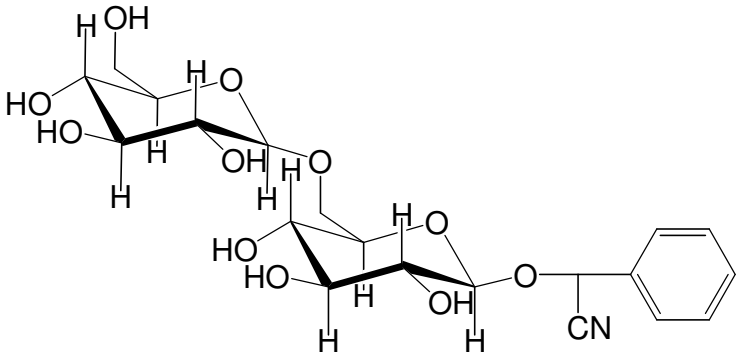
Function of glycosides in various species of plant have been revealed (24). Some glucosides are shown in Figure 3. They may, in times of dire need, release the sugar molecule to be used by the plant for energy or carbon sources, as well as the aglycone. Example is indican (indoxyl β-D-glucoside). It is a secondary metabolite found in indigo plant. When the leaves of indigo plant are broken, indican is immediately hydrolyzed to β-D-glucose and indoxyl by the action of β-glucosidase. This reaction is followed by the spontaneous conversion of free indoxyl by air oxidation into the natural dye, indigo (25). They may serve as a means of releasing toxicity. Since, aglycones are toxic to the plant in the free form, but when a sugar is attached to the molecule it is no longer toxic. Perhaps the aglycones are produced as a result of necessary metabolic activities of the plant and some means must be available to prevent their injury to the plant. Some glycosides protect the plant from infection. Plant disease organisms are often discouraged from invading, or otherwise damaging a plant because of the presence of certain glycosides in the plant.



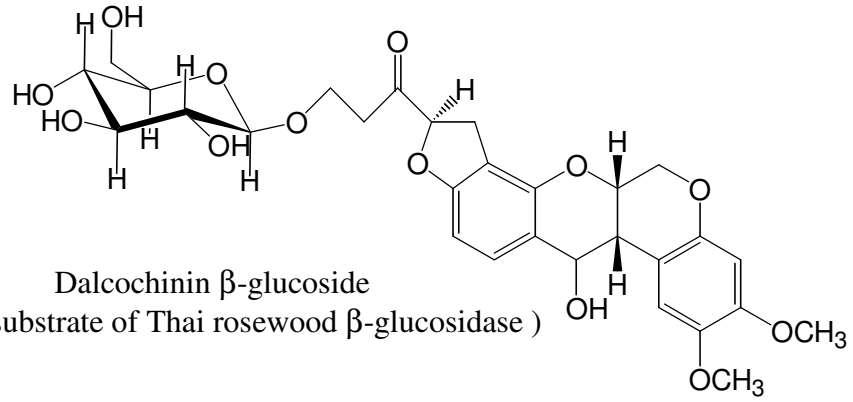
Salicin



Arbutin



Amygdalin



Dalcochinin β-glucoside
(natural substrate of Thai rosewood β-glucosidase)

Figure 3: Some β-glucosides.

1.3 *Plumeria* Species

Plumeria spp. are a tropical flowering tree, sometimes-called frangipani, in the plant family Apocynaceae. Though tropical by nature, when protected from frost, they are well suited to subtropical climates in the United States in states bordered by the Gulf of Mexico, and in Southern California. They are prolific in Hawaii. *Plumeria* are valued as landscape plants, ornamentals, and for their flowers. The flowers come in seemingly endless variety of color, size and fragrance.

There are numerous reports on the ethnomedical use of plants belonging to the pantropic genus *Plumeria*. In South America, decoctions of leaves, bark or heartwood are used as an anthelmintic, purgative, emmenagogue, and for the topical treatment of various afflictions of the skin (26). Extracts of various *Plumeria* spp. have been shown to exhibit significant antibacterial, antifungal and antiviral activity (27). The iridoid glucosides have been isolated from the bark of *Plumeria rubra* of Indonesia origin (28). This purified β-glucoside has the structure shown in Figure 4.

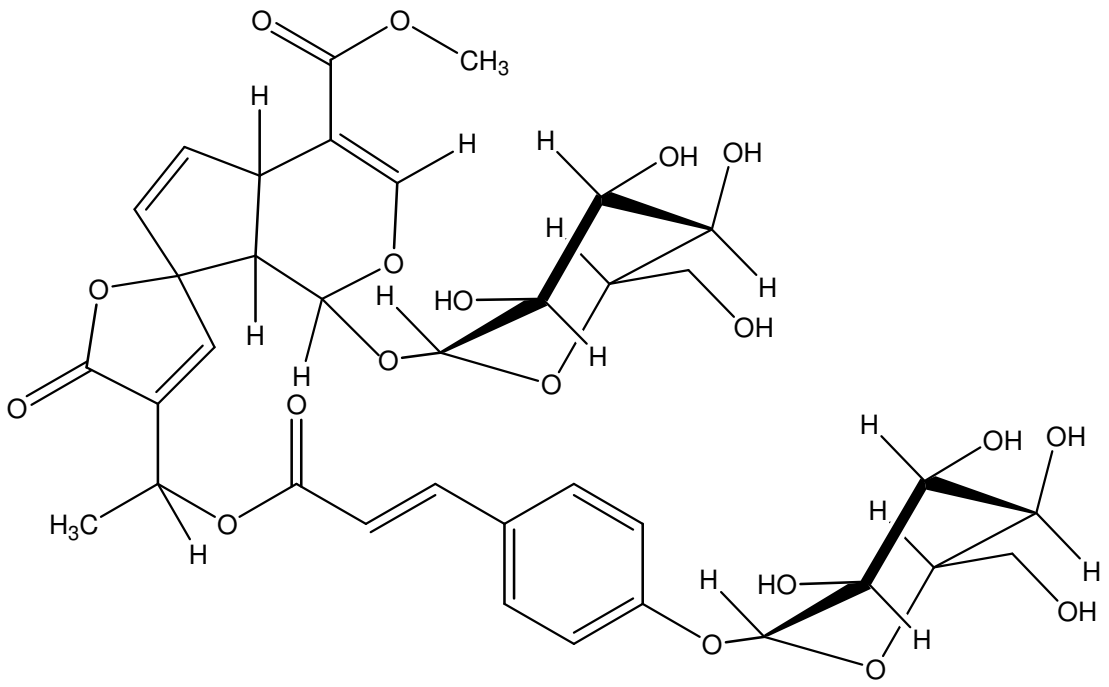


Figure 4: Structure of iridoid glucoside isolated from the bark of *Plumeria rubra* of Indonesia origin.

1.4 Aim of this Thesis

Since β-glucosidases are useful in various applications and they are widely different in substrate specificity, a novel β-glucosidase is of interest. This thesis aims to screen for β-glucosidase and its natural substrate from a new plant. After that, the β-glucosidase and its β-glucoside will be purified and characterized.

CHAPTER II
MATERIALS AND METHODS

2.1 Materials

Bio-Rad protein assay dye concentrate was purchased from Bio-Rad Laboratories, Hercules, CA, USA. Bovine serum albumin (BSA), p-nitrophenyl-β-D-glucopyranoside (pNPGlc), p-nitrophenyl-β-D-manopyranoside (pNPMan), p-nitrophenyl-β-D-fucopyranoside (pNPFuc), p-nitrophenyl-β-D-galactopyranoside (pNPGal), p-nitrophenyl-α-D-glucopyranoside, p-nitrophenyl-α-L-fucopyranoside, p-nitrophenol (pNP), glucose, methyl-β-D-glucopyranoside (MGlc), cellobiose (β-1,4) and gentiobiose (β-1,6) were bought from Sigma Co. Ltd.. Citric acid, di-sodium hydrogen phosphate (Na_2HPO_4), di-sodium carbonate (Na_2CO_3), methanol, ethanol, 1-propanol, 2-propanol, 1-butanol and 2-butanol and silica gel 60 F_{254} aluminium TLC sheets were purchased from Merck. Glucose kit (for determining glucose release) was bought from BML. Sephadex LH-20 and all materials for SDS-PAGE were purchased from Pharmacia Biotech, except that glycine and N, N, N', N'-tetramethylethylenediamine (TEMED) were from Amersham. Ethylacetate (EtAc) and deuterium oxide (D_2O) were bought from Fluka.

2.2 Screening for β-glucosides and β-glucosidases in Thai plants

2.2.1 Screening for β-glucosides in Thai plants

Plant tissues were homogenized with ethanol (4 ml buffer per gram tissues) and keep at 4 ^{o}C for overnight. After that, the ethanol extract is filtered through filter paper and the supernatant is concentrated by rotary evaporation to give the crude substrate. Screening for β-glucosides was performed by incubating crude substrate from each plant tissues with the β-glucosidase from Thai rosewood, almond or cassava-stem in 0.1M sodium acetate buffer pH 5.0 at 37 ^{o}C for overnight. The products were separated

by TLC (butanol: ethanol: water = 5: 3: 2 as a mobile phase) and detected by UV or staining with 10% sulfuric acid in ethanol.

2.2.2 Screening for β-glucosidase enzymes using different substrates

Plant tissues were homogenized with the extraction buffer containing 0.1 M sodium acetate pH 5.5, 1 mM PMSF and 5% PVPP (2 ml buffer per gram tissue). The extract was filtered through 4 layers of gauze and centrifuge at 10,000 rpm for 30 min at 4 ^{o}C to give the supernatant. Dowex 2X-8 resin was added to eliminate phenolic compounds. After removing Dowex 2X-8 resin from the extract by suction, the supernate was centrifuged at 10,000 rpm for 30 min at 4 ^{o}C to give the crude enzyme.

β-glucosidase enzyme was screened for by incubating with 2 mM arbutin or the crude natural substrate in 0.1 M sodium acetate buffer pH 5.5 at 37 ^{o}C for overnight. Products were visualized on the TLC plate by observeing under UV or staining with 10% sulfuric acid in ethanol.

In screening for β-glucosidase by using pNP-Glc as the substrate, the crude extract of each plant tissue was incubated with 1 mM pNP-Glc in buffer (0.1 M sodium acetate buffer pH 5.5) at 37 ^{o}C for 30 min. 2 M Na_2CO_3 is added to stop the reaction and released pNP was measured by spectrophotometer at 400 nm.

2.3 Isolation and purification of β-glucosidase and its substrate from the flowers of *Plumeria obtusa* Linn

2.3.1 Purification of β-glucosides from the flowers of *Plumeria obtusa* Linn

2.3.1.1 Ethanol extraction of β-glucoside from the flowers of *Plumeria obtusa* Linn.

The fresh flowers (250 g) of *Plumeria obtusa Linn* were homogenized by using a mortar and pastle in liquid nitrogen and transferred to a beaker containing absolute ethanol (4ml.buffer per gram tissues). The extract was boiled to eliminate β-glucosidase activity and stirred at 4 ^{o}C for overnight. The ethanol extract was filtered

through filter paper and concentrated by rotary evaporation to give a yellow syrup as crude substrate.

2.3.1.2 Purification of β-glucosides from the flowers of *Plumeria obtusa* Linn

The 10 g of crude substrate was chromatographed on a Sephadex LH-20 column (460 ml. column volume) equilibrated with methanol. The column was eluted by methanol as mobile phase. Fractions from the column were analyzed by silica gel TLC (chloroform:methanol = 3:1). The β-glucoside containing fraction was pooled and concentrated by rotary evaporation for the next step of purification. After that, the pool of β-glucoside containing fraction was purified by preparative TLC (silica gel 60G F_{254}) using chloroform:methanol (3:1) as a mobile phase and observed under UV 254 nm. The β-glucoside band (the most intense band under UV light) was scraped from TLC plate. MeOH was added to dissolve the substrate and silica gel was removed by suction to give 200 mg of purified β-glucoside.The structure of β-glucoside was established by spectroscopic data.

2.3.1.3 TLC analysis of purified β-glucoside

Samples were applied on a TLC plate (silica gel 60 F_{254}, 0.2 mm, aluminum sheet) at 1 cm from the bottom edge of the plate. Developing solvent was prepared by mixing chloroform/methanol (3:1 by volume). The development chamber was equilibrated with the developing solvent for 30 min to saturate the atmosphere of the chamber with solvent vapor. The spotted TLC plated was developed in the chamber until the solvent front had run a distance of about 90% of total height of TLC plate. The TLC plate was dried with a hair drier, and sample bands were visualized under UV light, or by dipping with 10% sulfuric acid in absolute ethanol and heating at 125 ^{o}C for 5 min.

2.3.1.4 Determination of β-glucoside from the flowers of *Plumeria obtusa* Linn by TLC

The presence of β-glucoside was detected by digesting fractions from the column with its hydrolytic enzyme, so that the digested product will have changed mobility on the TLC plate. So, the position of band that disappears after digestion is β-glucoside from the flower of *Plumeria obtusa* Linn.

Reaction mixtures containing 20 µl of samples and 80 µl of purified β-glucosidase from *P. obtusa* L, with final volume 100 µl and incubating at 37 ^{o}C overnight. After the reaction, samples were analyzed by TLC on a silica gel 60 F_{254}, aluminum sheet as described above.

2.3.2 Purification of β-glucosidase from the flowers of *Plumeria obtusa* Linn.

2.3.2.1 Preparation of Crude Enzyme

The fresh flowers (1kg) of *P.obtusa* L. were homogenized immediately after collection by immersing in liquid nitrogen. Then extraction buffer containing 0.05 M sodium acetate pH 5.5 containing 1 mM phenylmethylsulfonyl fluoride (PMSF) to inhibit proteolysis, and 5% (w/v) polyvinylpolypyrrolidone (PVPP) to eliminate polyphenolic substances, is added (2 ml buffer/ gram tissue). The extract was filtered through 4 layers of gauze. The homogenates were centrifuged at 10,000 rpm for 30 minutes to give the supernatant. Dowex 2X-8 resin was then added to eliminate phenolic compounds which interfere with both glycosidase activity and protein determination, followed by gentle stirring for 1 hour at 4°C and removal by suction. The solution was centrifuged at 10,000 rpm for 30 minutes to give the supernatant as the crude extract. All experiments are carried out at 4 °C.

2.3.2.2 Ammonium sulfate precipitation

The crude extract is fractionated by adding solid $(NH_4)_2SO_4$ to 75% saturation and stirred at 4 °C overnight. The precipitate is collected by centrifugation and redissolved in a small volume of 0.1 sodium acetate pH 5.5. After that, the precipitate

was dialyzed against 10 mM sodium phosphate buffer pH 7.0 at 4 °C for overnight before chromatography on a DEAE-cellulose column.

2.3.2.3 Column chromatography

The dialyzed precipitate is then separated on various types of column chromatography to give the purified enzyme.

2.3.2.3.1 Anion-exchange Chromatography

The dialyzed precipitate was centrifuged at 5000 rpm for 30 min before chromatography on a DEAE-cellulose column (120 ml column volume) which was equilibrated with 10 mM sodium phosphate buffer pH 7.0.To wash unbound protein, 10 mM sodium phosphate buffer pH 7.0 is used as mobile phase. Then bound protein was eluted by performing a linear gradient of 0-0.3 M NaCl in 10 mM sodium phosphate buffer pH 7.0. Each fraction was collected in 3.0 ml, and used for measurement of conductivity, assayed for β-glucosidase activity and protein concentration at A_{280}.

2.3.2.3.2 Affinity Chromatography

Fractions contain β-glucosidase activity from DEAE-cellulose were pooled and concentrated by centriprep (30 KD cut off) before loading on a Con A-Sepharose column (30 ml column volume). The column was equilibrated with 0.5 M NaCl in 0.02 M sodium phosphate pH 7.0 and eluted with 0.3 M of α-methylmannoside. Each fraction was collected in 3.0 ml volume, assayed for β-glucosidase activity and protein concentration at A_{280}.

2.3.2.3.3 Gel Filtration

The pooled fractions containing β-glucosidase activity from Con A-Sepharose was concentrated by centriprep (30 KD cut off) to a volume of 7.0 ml. The concentrated sample was directly applied on a Sephacryl S-300 column (400 ml. column volume), equilibrated with 0.15 M NaCl in 0.1 M sodium acetate buffer pH 5.5. The column was run with equilibrating buffer at a constant flow rate of 0.5

ml/min. Fraction was collected in 3.0 ml, assayed for β-glucosidase activity and measured the protein content at A_{280}.

2.3.2.3.4 Hydrophobic Interaction Chromatography

Next the Sephacryl-pool was concentrated to a volume of 5.0 ml using a centriprep (30 KD cut off). $(NH_4)_2SO_4$ was added to the pool obtained from Sephacryl S-300 column to a concentration of 1.5 M. The sample was chromatographed on a Butyl-Sepharose 4 fast flow (7 ml column volume), equilibrated with 1.5 M $(NH_4)_2SO_4$ in 0.05 M sodium phosphate buffer pH 7.0. Then bound protein was eluted by performing a linear gradient of 1.5 - 0 M $(NH_4)_2SO_4$ in 0.05 M sodium phosphate buffer pH 7.0. Each fraction was collected in 2.0 ml, and used for measurement of conductivity, β-glucosidase activity and protein concentration by A_{280}.

2.3.3 β-Glucosidase activity assay

2.3.3.1 By pNP release

β-glucosidase activity was assayed as p-nitrophenol (pNP) releasing activity by hydrolysis of pNP-Glc. The reaction mixture contained enzyme solution, pNP-Glc in 0.1 M sodium acetate buffer pH 5.5. The enzyme solution (50µl) was added to sodium acetate buffer pH 5.5 (850 µl) and pre-incubated at 40 ^oC for a few minutes. The reaction was started by adding 100 µl of 50 mM pNP-Glc, to a final concentration of 5 mM, and further incubated for 30 min. The reaction was stopped by adding 2 ml of 2 M sodium carbonate (Na_2CO_3) solution, and the released pNP was measured with a spectrophotometer at a wavelength 400 nm. The autohydrolysis of the substrate was determined in the same manner using the buffer instead of the enzyme.

2.3.3.2 By glucose release

β-glucosidase activity was also determined as glucose releasing activity by hydrolysis of disaccharides or glucosides. The reaction mixture (100 µl) is composed of enzyme solution, natural substrate of β-glucosidase in 0.1 M sodium acetate buffer pH 5.5. The enzyme solution (80 µl) was incubated with the purified β-glucoside from

Plumeria obtusa Linn (20µl) dissolved in 0.5 M sodium acetate buffer, pH 5.5 at 37 ^{o}C for 30 min. The reaction was stopped by boiling for 5 min and added with 1 ml glucose assay kit. Glucose release was measured with a spectrophotometer at wavelength 505 nm.

2.3.4 Protein determination

The Bio-Rad protein assay reagent (200 µl) was added to 800 µl of diluted enzyme solution, left for 5 min, and the absorbance at 595 nm was measured with a spectrophotometer. Standard curve of protein was prepared with bovine serum albumin (BSA), in the range of 2-10 µg BSA. For the chromatography, the protein content was measured by A_{280}.

2.3.5 Enzyme purity

The purity of all enzymes was detected by discontinuous 10% SDS-PAGE (8 cm x 7 cm x 0.1 cm) with 3% acrylamide stacking gel (8 cm x 1.5 cm x 0.1 cm) on a slab gel system according to the Protein Electrophoresis technical manual (29). The samples were mixed with an equal volume of sample buffer volume and boiled for 3 min prior to application onto the gel. Electrophoresis was performed at a constant voltage of 100 V. Electrode buffer contained 0.025 M Tris, 0.192 M glycine pH 8.3 and 0.1% (w/v) SDS. After that, the gel was stained with Coomassie blue R-250. Each protein band was compared to molecular weight standard (212.00 KDa to 2.34 KDa), including rabbit muscle myosin, *E.coli* maltose-binding protein-β-galactosidase, *E.coli* β-galactosidase, rabbit muscle phosphorylase b, bovine serum albumin, bovine liver glutamic dehydrogenase, *E.coli* maltose-binding protein 2, porcine muscle lactate dehydrogenase M, rabbit muscle triosephosphate isomerase, soybean trypsin inhibitor, chicken egg white lysozyme, bovine lung aprotinin and bovine pancreas insulin A, B chain, from BioLabs, Inc.

2.4 Study of properties of β-glucosidase enzyme and its natural substrate from the flowers of *Plumeria Obtusa* Linn

2.4.1 Physical properties and characterization of the purified β-glucosidase enzyme

2.4.1.1 Native molecular weight of β-glucosidase by gel filtration

The bound fractions of Con A-Sepharose were applied to a Sephacryl S-300 column equilibrated with 0.15 M NaCl in 0.1 M sodium acetate buffer pH 5.5. The column was calibrated with thyroglobulin, ferritin, catalase and aldolase (3 mg/ml). To determine the native molecular weight of enzyme, the Ve/Vo of each standard marker was plotted against log molecular weight of the protein.

2.4.1.2 Agarose isoelectric focusing

Isoelectric focusing (IEF) was performed in a Bio-Rad minigel IEF apparatus. The agarose IEF gel was prepared from 1 % agarose, 5 % sorbitol, 10 % glycerol, 2 % ampholyte (Ampholine pH range 3.5/10). The solution of 5 ml agarose gel was prepared by weighing 0.05 g agarose, 0.25 g sorbitol, 0.5 ml anhydrous glycerol and 2.25 ml distilled water. The gel solution was slowly stirred and heated until dissolved in boiling water. The gel was left to cool down until the temperature is about 85 ^{o}C. Then the 40% Ampholine pH range 3.5/10 (0.25 ml) and 2 ml of distilled water were added while stirring. Next the gel was poured on to a pre-warm 55 ^{o}C casting IEF tray. The IEF agarose gel was left at room for 1 hr on a flat space before putting it into the refrigerator and being left there overnight. For running, the sample (4 – 8 μl) was applied onto the gel with an applicator. The gel was run at 100 V for 15 min, 200 V for 15 min, and 450 V for an additional 60 min in the cold room. After running, the IEF gel was stained with 4-methylumbelliferyl-β-D-glucopyranoside (4-MU-Glc) as described in section 2.4.1.6 for the detection of β–glucosidase activity. In addition, for standard pI protein, the gel was fixed for protein staining by immersing it in fixative solution (30 % methanol, 5 % trichloroacetic acid and 3.5 % sulfosalicylic acid) for 15 min. Then the gel was transferred to 95 % ethanol for 30 min for background

clearance. After the ethanol wash, the gel was placed on to a glass plate and covered with a piece of filter paper soaked with ethanol. About 8-10 sheets of dry filter papers were placed on the top of the soaking paper and a 1 kg weight was placed over for 30 min. Then the gel was removed, dried with an air blower, and stained for protein with 0.2 % coomassie brilliant blue R-250 in 28 % isopropanol and 14 % acetic acid for 1 h. The gel was further destained for 30 min with 28 % isopropanol and 14 % acetic acid.

2.4.1.3 pH optima of β-glucosidase from the flowers of *Plumeria obtusa* Linn

The β-glucosidase from section 2.3.2.3.3 was used to assay for activity at different pH using pNP-Glc. The enzyme (0.05ml) was pre-incubated with 0.65 ml of citrate-phosphate buffer pH 4.0-7.5 for a few minutes at 40 ^{o}C and then 0.3 ml of 0.05 M pNP-Glc dissolved in distilled water was added. The reaction was further incubated for 30 min before the addition of 1 ml of 2 M sodium carbonate solution to stop the reaction. Then the released pNP was measured with a spectrophotometer at wavelength 400 nm. The β-glucosidase was also assay for activity at pH 4.0-7.5 using purified β-glucoside from the flowers of *Plumeria obtusa* Linn. The enzyme (0.02 ml) was incubated with its natural substrate dissolved in citrate-phosphate buffer pH 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0 or 7.5.(0.08 ml). The reaction was incubated for 30 min at 40 ^{o}C. Then the reaction was stopped by boiling for 5 min and added with 1 ml glucose assay kit. Glucose release was measured with a spectrophotometer at wavelength 505 nm. The pH of the reaction was also checked by measuring the pH of mixture of 0.05 ml of 0.1 M sodium acetate buffer pH 5.5, 0.3 ml of distilled water, 0.65 ml citrate-phosphate buffer pH 4.0 – 7.5. Then the activity at each pH value of β-glucosidase was plotted against the pH value.

2.4.1.4 Kinetics study of purified β-glucosidase from the flowers of *Plumeria obtusa* Linn

2.4.1.4.1 With pNP-Glc

The enzyme obtained from section 2.3.2.3.3 was incubated at 40 °C with 0.1 ml of 50 mM (5 mM final concentration) pNP-Glc and 0.1 M sodium acetate buffer, pH 5.5 for 10-60 min. At time 10, 20, 30, 40, 50, and 60 min, 1 ml of 2 M sodium carbonate was added to stop the reaction. Then the sample was measured at A_{400} . To determine the initial velocity and suitable reaction time of the enzyme, the amount of pNP (measured at A_{400}) was plotted against the incubation time. The enzyme was assayed at pH 5.5, 40 °C using various concentration of pNP-Glc (0.2-20 mM) in 0.1 M sodium acetate buffer for 30 min. The reaction was stopped with addition of 1 ml of 2 M sodium carbonate solution and the absorbance at 400 nm measured to calculate the amount of the pNP produced.

2.4.1.4.2 With β-glucoside from the flowers of *Plumeria Obtusa* Linn as substrate

The enzyme obtained from section 2.3.2.3.3 (0.02 ml) was incubated at 40 °C with 0.08 ml of 5 mM β-glucoside in 0.1 M sodium acetate buffer pH 5.5 at a final volume of 0.1 ml for 5 min – 60 min. The reaction was stopped by boiling at 100 °C for 5 min at time 5, 10, 20, 30, 40, 50 and 60 min. Then the product liberated, was quantitated by measuring glucose released as described in section 2.3.3.2. To determine the initial velocity and suitable reaction time of enzyme, the concentration of glucose produced at each time plotted against the incubation time. The enzyme was assayed at pH 5.5, 40 °C using various concentration of β-glucoside from the flowers of *Plumeria obtusa* Linn (2-16 mM) in 0.1 M sodium acetate buffer for 30 min. The reaction was stopped by boiling at 100 °C for 5 min. Then glucose released was measured at A_{505} as described in section 2.3.3.2.

2.4.1.4.3 Determination of K_m and V_{max}

K_m and V_{max} of β-glucosidase with either pNP-Glc and β-glucoside from the flowers of *Plumeria obtusa* Linn as substrate was determined by the Michaelis-Menten plots using Prism 3 program.

2.4.1.5 Vary substrate of hydrolysis

Various glucoside substrates, namely pNP-β-D-glucose, pNP-β-D-fucose, pNP-β-D-mannose, pNP-β-D-galactose, pNP-α-L-fucose and pNP-α-D-glucose were used to compare hydrolytic activity and detected activity by pNP release (section 2.3.3.1). The final substrate concentration of the reaction was 5 mM. Other glucoside substrates such as Prunasin, Torvoside H, Linamarin, Dalchochinin-β-glucoside, Esculin, Arbutin, Gentiobiose, Amygdalin, Salicin, 4-MU-β-D-glucoside, Methyl-β-D-glucoside, Hexyl-β-D-glucoside were used to compare hydrolytic activity with natural substrate from *P.obtusa* L. flowers and detected activity by glucose release (section 2.3.3.2). The final substrate concentration of the reaction was 4 mM

2.4.1.6 Determination of β-glucosidase activity using fluorescent substrate.

The gel from non-denaturing electrophoresis or isoelectric focusing was detected for β-glucosidase activity using 4-methylumbelliferyl-β-D-glucopyranoside (4-MU-Glc).The gel was soaked in 1 mM 4-MU-Glc dissolved in 0.1 M sodium acetate buffer pH 5.5 and shaken at 37 ^{o}C for 10 min. Bright purplish fluorescent bands of methylumbelliferone released were detected under ultraviolet light and photograph by GEL-DOC .

2.4.2 Study of natural substrate from *Plumeria obtusa* Linn flowers.

2.4.2.1 Structure elucidation of β-glucoside from the flowers of *Plumeria obtusa* Linn

The purified β-glucoside from section 2.3.1.2 (about 30 mg) was studied by NMR to establish the structure. The sample prepared in D_2O was analysed by a Bruker WM-400 spectrometer.

2.4.2.2 Structure elucidation of aglycone after digested with purified β-glucosidase from the flowers of *Plumeria obtusa* linn

β-glucoside (50 mg) was incubated with purified β-glucosidase from the flowers of *Plumeria obtusa* Linn in 0.1 M sodium acetate buffer pH 5.5, 40^{o}C for overnight. After evaporation of the solvent, the reaction mixture was separated on TLC plate. Chloroform:methanol (3:1) was used as a mobile phase and the TLC was observed under UV 254 nm.The aglycone band was scraped from TLC plate. MeOH was added to dissolve the substrate and silica gel was removed by suction. The structure of aglycone was established by spectroscopic measurement.

CHAPTER III
RESULTS

3.1 Screening for β-glucosides and β-glucosidases in Thai plants

3.1.1 Screening for β-glucosides

In preliminary studies, β-glucosides were found in many Thai plant species. The appearance of β-glucosides was detected by disappearance of bands after digestion with β-glucosidase, and appearance of new bands with high mobility in TLC plate (normal phase). In our experiment, β-glucosides were screened for in Thai plants from 15 species. It was found that the crude extracts from 8 plant species contained β-glucosides (*Plumeria obtusa* Linn, *Plumeria rubra* Linn,. *Murraya paniculata* Jack, *Nerium indicum* Mill, *Oroxylum indicum* Vent, *Tecoma stans* Juss, *Artabotrys hexapetalus* (Linn.f.) *Bhandari, Rhinacanthus nasutus* KurZ) The results were shown in Fig 5-12. β-glucosidase from different sources are different in substrate specificity, so I used 3 β-glucosidases from Thai rosewood, almond and cassava-stem to screen for β-glucosides.

The results shown the extracts from stalks of *Plumeria obtusa* Linn (Lane 1 in Figure 5) have β-glucosides that could be detected by all three enzyme. Glucosides were detected by the disappearance of a band (⇨) after digestion with β-glucosidase, and the appearance of a more intense band with higher Rf at the point (➔) as shown in lane 2-4 in Figure 5. In lane 5, (Figure 5) shows the crude extract from the flowers of *Plumeria obtusa* Linn before digestion with the β-glucosidase. After digestion reaction, the band (⇨) disappears, and new bands appear at the arrow (➔ in lane 6-8, Figure 5). Similarly, the crude extracts from leaves of *Plumeria obtusa* Linn contained β-glucodise band (⇨ , Lane 9, Figure 5) that could be detected by Thai rosewood, almond, cassava-stem β-glucosidase. The more intense bands with high mobility are shown with an arrow (➔) in lane 10-12 (Figure 5).

The crude extract from leaves and flowers of *Plumeria rubra* Linn also showed the disappearance of a band (⇨ in lane 1 and 5 respectively) after digestion with 3 β-glucosidases, and the appearance of new bands with high mobility at the arrow (→, lane 2-4 and lane 6-8). Indicating that, the crude extracts from leaves and flowers of *Plumeria rubra* Linn contain β-glucoside detected by Thai rosewood, almond and cassava-stem β-glucosidase (Figure 6).

From Figure 7, lane 1 shows the crude extract from leaves of *Tecoma stan* Juss before digestion with β-glucosidase. After digestion, the band (⇨, lane 1) was disappears when digested with β-glucosidase from Almond but not when digested by other β-glucosidases (Thai rosewood and cassava-stem β-glucosidase). In lane 9, the crude extracts from the flowers of *Tecoma stan* Juss are shown before digestion with β-glucosidase. The band (⇨ , lane 9) disappears after digestion with β-glucosidase from Thai rosewood and almond but is poorly digested by cassava stem β-glucosidase. However, no β-glucoside is found in the extract of pods and stalks of *Tecoma stan* Juss (lane 6-8 and lane 14-16, respectively).

Similarly, the crude extract from the flowers of *Murraya paniculata* Jack (lane 10, Figure 8) contain β-glucoside represented by the arrow (⇨) because this band disappears after digestion with Thai rosewood and almond β-glucosidase (lane 11 and 12 in Figure 8 respectively).

From Figure 9, the crude extract from leaves of *Nerium indicum* Mill showed the disappearance of band (⇨ in lane 1) after digested with β-glucosidase from Thai rosewood and almond, and the appearance of new bands with high mobility at the arrow point (→, lane 2 and 3). The crude extract from stalks of *Nerium indicum* Mill contain β-glucoside represented by the arrow (⇨) because this band disappears after digestion with Thai rosewood β-glucosidase (lane 6). Similarly, the crude extract from roots, flowers and pods of *Nerium indicum* Mill also showed the disappearance of a band (⇨ in lane 9,13 and 17 respectively) after digestion with β-glucosidase from Thai rosewood and almond, and the appearance of new bands with higher mobility (→, lane 10-11, 14-15 and lane 18-19, respectively). The results show that the crude extracts from *Nerium indicum* Mill were poorly digested with cassava-stem β-glucosidase (lane 4, 8, 12, 16 and 20).

The crude extracts from stalks of *Oroxylum indicum* Vent showed the presence of β-glucoside because of the disappearance of band (⇨ , in lane1, Figure 10) after digestion with β-glucosidase from Thai rosewood and almond. The crude extracts from stalks of *Oroxylum indicum* Vent were poorly digested by cassava-stem β-glucosidase. β-glucoside could not detected from the extracts of leaves of *Oroxylum indicum* Vent by Thai rosewood, almond and cassava-stem β-glucosidase (lane 5-8, Figure 10).

From Figure 11, the crude extract from leaves of *Rhinacanthus nasutus* KurZ showed the disappearance of a band (⇨ , in lane 1) after digestion with β-glucosidase from Thai rosewood and almond, suggesting that the extract from leaves of this plant contain β-glucoside.

Figure 12 showed the crude extract from flowers, stalks and leaves of *Artabotrys hexapetalus* (Linn.f*) Bhandari* (lane 1,5 and 9 respectively). The band (⇨ , in lane 1,5 and 9) disappears after digestion with β-glucosidase from Thai rosewood and almond, indicating that the crude extracts from the flowers, stalks and leaves of *Artabotrys hexapetalus* (Linn.f*) Bhandari* contain β-glucoside detected by Thai rosewood and almond β-glucosidases.

A comparison of amount of β-glucosides in 15 plant species is given in Table 2. The digestion pattern of crude extracts containing β-glucosides by different enzymes are shown in (Fig 5-12) and summarized in Table 3.

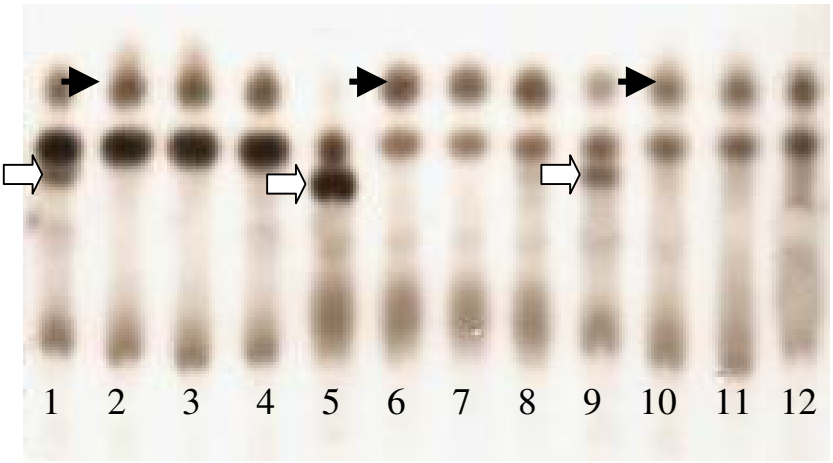


Figure 5: Crude extract from stalks, flowers and leaves of *Plumeria obtusa* L., before and after digestion with β-glucosidase.

(⇨) represent the β-glucoside band while (➧) represent the aglycone band after digestion with the enzyme.

Lane 1 = Crude extract from stalks of *Plumeria obtusa* L

Lane 2-4 = Crude extract from stalks of *Plumeria obtusa* L incubated with Thai rosewood, Almond and Cassava-stem β-glucosidase, respectively.

Lane 5 = Crude extract from flowers of *Plumeria obtusa* L

Lane 6-8 = Crude extract from flowers of *Plumeria obtusa* L incubated with Thai rosewood, Almond and Cassava-stem β-glucosidase, respectively.

Lane 9 = Crude extract from leaves of *Plumeria obtusa* L

Lane10-12 = Crude extract from leaves of *Plumeria obtusa* L incubated with Thai rosewood, Almond and Cassava-stem β-glucosidase, respectively.

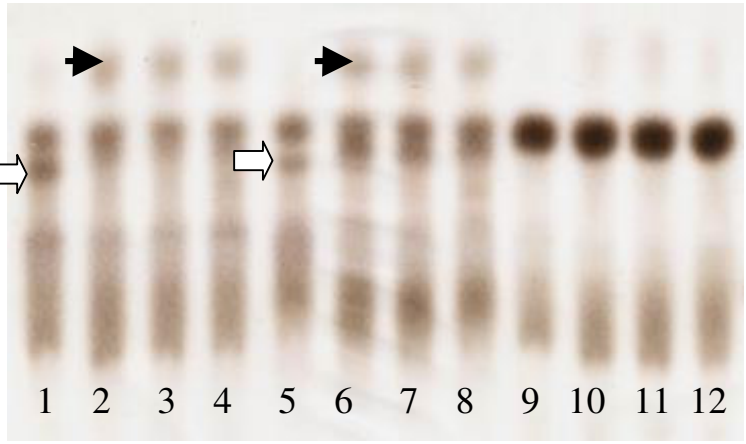


Figure 6: Crude extract from leaves, flowers and stalks of *Plumeria rubra* L., before and after digestion with β-glucosidase.

(⇨) represent the β-glucoside band while (➔) represent the aglycone band after digestion with the enzyme.

Lane 1 = Crude extract from leaves of *Plumeria rubra* L

Lane 2-4 = Crude extract from leaves of *Plumeria rubra* L incubated with Thai rosewood, Almond and Cassava-stem β-glucosidase, respectively.

Lane 5 = Crude extract from flowers of *Plumeria rubra* L

Lane 6-8 = Crude extract from flowers of *Plumeria rubra* L incubated with Thai rosewood, Almond and Cassava-stem β-glucosidase, respectively.

Lane 9 = Crude extract from stalks of *Plumeria rubra* L

Lane10-12 = Crude extract from stalks of *Plumeria rubra* L incubated with Thai rosewood, Almond and Cassava-stem β-glucosidase, respectively.

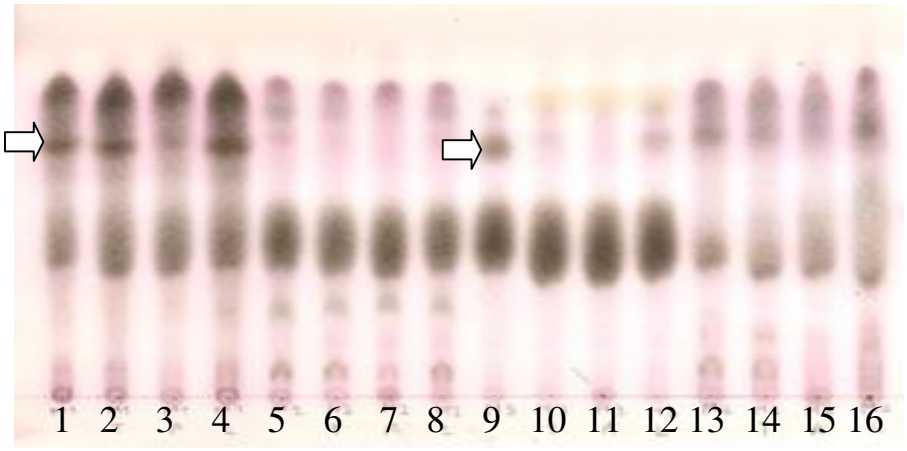


Figure 7: Crude extract from leaves, pods, flowers, and stalks of *Tecoma stans* Juss, before and after digestion with β-glucosidase.

(⇨) represent the β-glucoside band.

Lane 1 = Crude extract from leaves of *Tecoma stans* Juss

Lane 2-4 = Crude extract from leaves of *Tecoma stans* Juss incubated with Thai rosewood, Almond and Cassava-stem β-glucosidase, respectively.

Lane 5 = Crude extract from pods of *Tecoma stans* Juss

Lane 6-8 = Crude extract from pods of *Tecoma stans* Juss incubated with Thai rosewood, Almond and Cassava-stem β-glucosidase, respectively.

Lane 9 = Crude extract from flowers of *Tecoma stans* Juss

Lane10-12 = Crude extract from flowers of *Tecoma stans* Juss incubated with Thai rosewood, Almond and Cassava-stem β-glucosidase, respectively.

Lane 13 = Crude extract from stalks of *Tecoma stans* Juss

Lane14-16 = Crude extract from stalks of *Tecoma stans* Juss incubated with Thai rosewood, Almond and Cassava-stem β-glucosidase, respectively.

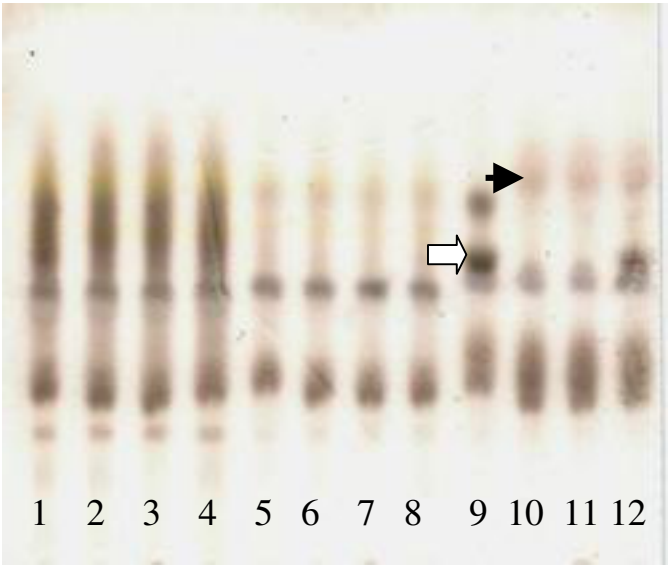


Figure 8: Crude extract from stalks, leaves and flowers of *Murraya paniculata* Jack, before and after digestion with β-glucosidase.

(⇨) represent the β-glucoside band while (➔) represent the aglycone band after digestion with the enzyme.

Lane 1 = Crude extract from stalks of *Murraya paniculata* Jack

Lane 2-4 = Crude extract from stalks of *Murraya paniculata* Jack incubated with Thai rosewood, Almond and Cassava-stem β-glucosidase, respectively.

Lane 5 = Crude extract from leaves of *Murraya paniculata* Jack

Lane 6-8 = Crude extract from leaves of *Murraya paniculata* Jack incubated with Thai rosewood, Almond and Cassava-stem β-glucosidase, respectively.

Lane 9 = Crude extract from flowers of *Murraya paniculata* Jack

Lane10-12 = Crude extract from flowers of *Murraya paniculata* Jack incubated with Thai rosewood, Almond and Cassava-stem β-glucosidase, respectively.

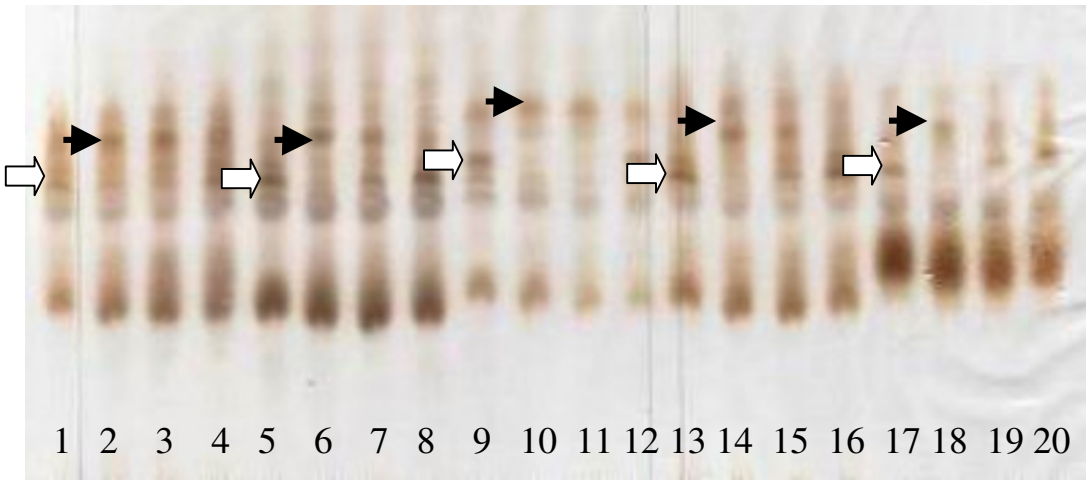


Figure 9: Crude extract from leaves, stalks, roots, flowers and pods of *Nerium indicum* Mill, before and after digestion with β-glucosidase.

(⇨) represent the β-glucoside band while (➔) represent the aglycone band after digestion with the enzyme.

Lane 1 = Crude extract from leaves of *Nerium indicum* Mill

Lane 2-4 = Crude extract from leaves of *Nerium indicum* Mill incubated with Thai rosewood, Almond and Cassava-stem β-glucosidase, respectively.

Lane 5 = Crude extract from stalks of *Nerium indicum* Mill

Lane 6-8 = Crude extract from stalks of *Nerium indicum* Mill incubated with Thai rosewood, Almond and Cassava-stem β-glucosidase, respectively.

Lane 9 = Crude extract from roots of *Nerium indicum* Mill

Lane10-12 = Crude extract from roots of *Nerium indicum* Mill incubated with Thai rosewood, Almond and Cassava-stem β-glucosidase, respectively.

Lane 13 = Crude extract from flowers of *Nerium indicum* Mill

Lane14-16 = Crude extract from flowers of *Nerium indicum* Mill incubated with Thai rosewood, Almond and Cassava-stem β-glucosidase, respectively.

Lane 17 = Crude extract from pods of *Nerium indicum* Mill

Lane18-20 = Crude extract from pods of *Nerium indicum* Mill incubated with Thai rosewood,

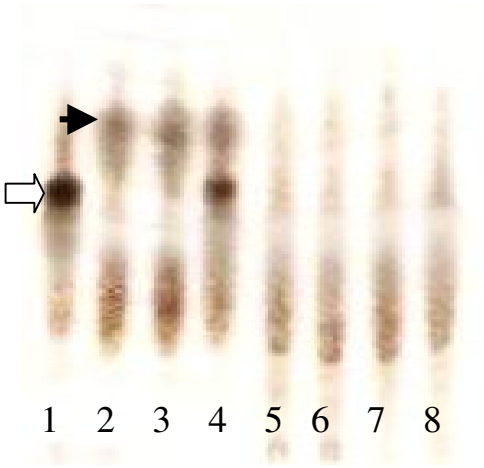


Figure 10: Crude extract from stalks and leaves of *Oroxylum indicum* Ven*t*, before and after digestion with β-glucosidase.

(⇨) represent the β-glucoside band while (➧) represent the aglycone band after digestion with the enzyme.

Lane 1 = Crude extract from stalks of *Oroxylum indicum* Vent

Lane 2-4 = Crude extract from stalks of *Oroxylum indicum* Vent incubated with Thai rosewood, Almond and Cassava-stem β-glucosidase, respectively.

Lane 5 = Crude extract from leaves of *Oroxylum indicum* Vent

Lane 6-8 = Crude extract from leaves of *Oroxylum indicum* Vent incubated with Thai rosewood, Almond and Cassava-stem β-glucosidase, respectively.

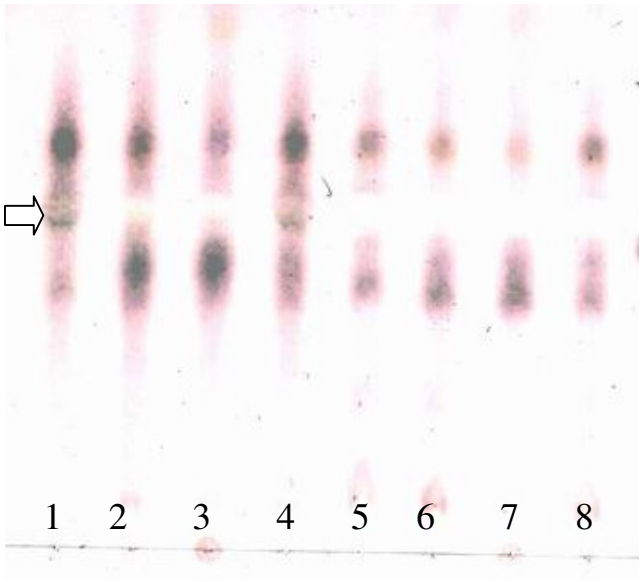


Figure 11: Crude extract from leaves and stalks of *Rhinacanthus nasutus* KurZ, before and after digestion with β-glucosidase.

(⇨) represent the β-glucoside band.

Lane 1 = Crude extract from leaves of *Rhinacanthus nasutus* KurZ

Lane 2-4 = Crude extract from leaves of *Rhinacanthus nasutus* KurZ incubated with Thai rosewood, Almond and Cassava-stem β-glucosidase, respectively.

Lane 5 = Crude extract from stalks of *Rhinacanthus nasutus* KurZ

Lane 6-8 = Crude extract from stalks of *Rhinacanthus nasutus* KurZ incubated with Thai rosewood, Almond and Cassava-stem β-glucosidase, respectively.

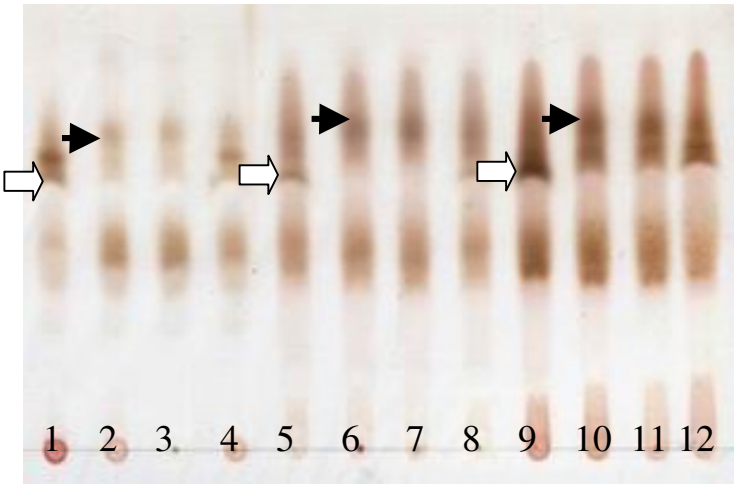


Figure 12: Crude extract from flowers, stalks and leaves of *Artabotrys hexapetalus* (Linn.f.) *Bhandar*i, before and after digestion with β-glucosidase.

(⇨) represent the β-glucoside band while (➔) represent the aglycone band after digestion with the enzyme.

Lane 1 = Crude extract from flowers of *Artabotrys hexapetalus* (Linn.f.) *Bhandar*i

Lane 2-4 = Crude extract from flowers of *Artabotrys hexapetalus* (Linn.f.) *Bhandar*i incubated with Thai rosewood, Almond and Cassava-stem β-glucosidase, respectively.

Lane 5 = Crude extract from stalks of *Artabotrys hexapetalus* (Linn.f.) *Bhandar*i

Lane 6-8 = Crude extract from stalks of *Artabotrys hexapetalus* (Linn.f.) *Bhandar*i incubated with Thai rosewood, Almond and Cassava-stem β-glucosidase, respectively.

Lane 9 = Crude extract from leaves of *Artabotrys hexapetalus* (Linn.f.) *Bhandar*i

Lane10-12 = Crude extract from leaves of *Artabotrys hexapetalus* (Linn.f.) *Bhandar*i incubated with Thai rosewood, Almond and Cassava-stem β-glucosidase, respectively.

Species	Leaves		Stalks		Flowers		Pods		Roots		Fruits	
	UV	H_2SO_4	UV	H_2SO_4	UV	H_2SO_4	UV	H_2SO_4	UV	H_2SO_4	UV	H_2SO_4
P. obtusa Linn	++	++	++	++	+++	+++	ND	ND	ND	ND	ND	ND
P. rubra Linn	++	++	-	-	+	+	ND	ND	ND	ND	ND	ND
M. paniculata Jack	-	-	+	-	+++	++	ND	ND	ND	ND	ND	ND
N. indicum Mill	+	+	++	++	+	+	+	+	+	+	ND	ND
R. nasutus KurZ	+	+	-	-	ND	ND	ND	ND	ND	ND	ND	ND
A. Hexapetalus Bhandari	-	+	-	+	-	+	ND	ND	ND	ND	ND	ND
T. stans Juss	+	++	-	-	+++	++	-	-	ND	ND	ND	ND
O. indicum Vent	-	-	+++	+++	ND	ND	ND	ND	ND	ND	ND	ND
V. heynii Spreng	-	-	-	-	ND	ND	ND	ND	ND	ND	ND	ND
S. venosa(Blume) *Spreng*	-	-	-	-	ND	ND	ND	ND	-	-	ND	ND
A.auriculiformis Cunn	-	-	-	-	-	-	-	-	ND	ND	ND	ND
S. sanitwongsci Craib	-	-	-	-	-	-	ND	ND	ND	ND	ND	ND
E. sphaericus	-	-	-	-	ND	ND	ND	ND	ND	ND	-	-
C. asiatica Linn	-	-	-	-	ND	ND	ND	ND	ND	ND	ND	ND
S. asper Lour	-	-	-	-	ND	ND	ND	ND	ND	ND	ND	ND

Table 2: Glucosides content in Thai plants detected by UV_{254} and H_2SO_4.

High glucoside content : (+++), medium glucoside content : (++), low glucoside content : (+) , no glucosides : (-) and no data available : ND

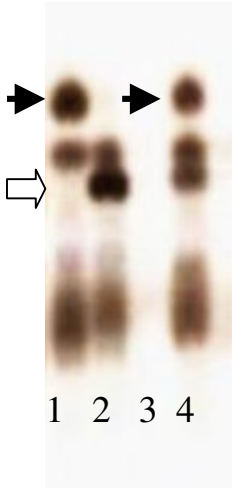
Species	Leaves			Stalks			Flowers			Pods			Roots			Fruits		
	T	A	C	T	A	C	T	A	C	T	A	C	T	A	C	T	A	C
P. obtusa Linn	+++	+++	+++	+++	+++	+++	+++	+++	+++	ND	ND	ND	ND	ND	ND	ND	ND	ND
P. rubra Linn	+++	+++	+++	-	-	-	+++	+++	+++	ND	ND	ND	ND	ND	ND	ND	ND	ND
M. paniculata Jack	-	-	-	-	-	-	+++	+++	++	ND	ND	ND	ND	ND	ND	ND	ND	ND
N. indicum Mill	+++	+++	-	+++	++	-	+++	++	-	+++	++	-	+++	+++	-	ND	ND	ND
R. nasutus KurZ	+++	+++	-	-	-	-	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
A. hexapetalus Bhandari	+++	+++	-	+++	+++	-	+++	+++	-	ND	ND	ND	ND	ND	ND	ND	ND	ND
T. stans Juss	NA	+++	-	-	-	-	+++	+++	++	-	-	-	ND	ND	ND	ND	ND	ND
O. indicum Vent	-	-	-	+++	+++	++	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
V. heynii Spreng	-	-	-	-	-	-	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
S. venosa(Blume)*Spreng*	-	-	-	-	-	-	ND	ND	ND	ND	ND	ND	-	-	-	ND	ND	ND
A. auriculiformis Cunn	-	-	-	-	-	-	-	-	-	-	-	-	ND	ND	ND	ND	ND	ND
S. sanitwongsci Craib	-	-	-	-	-	-	-	-	-	ND	ND	ND	ND	ND	ND	ND	ND	ND
E. sphaericus	-	-	-	-	-	-	ND	ND	ND	ND	ND	ND	ND	ND	ND	-	-	-
C. asiatica Linn	-	-	-	-	-	-	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
S. asper Lour	-	-	-	-	-	-	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND

Table 3: Digestion of plant glucosides by Thai Rosewood, almond and cassava-stem β-glucosidase.

Very good digestion: (+++), good digestion: (++), poor digestion: (+), no activity of digestion: (-), no data available: ND

3.1.2 Screening for β-glucosidase

From the results of β-glucoside screening, 6 plant species was screened for β-glucosidase enzyme by different substrate (pNP-Glc, arbutin and its natural substrate). The crude extract from plant tissues were assayed for β-glucosidase activity by pNP-Glc at pH 5.5, 40^{o}C.The activity is shown as nmole/min/g dry weight (Table 4). From the Table 4, comparing the β-glucosidase activity in each source, it was shown the sources that contained β-glucosidase activity are *Plumeria obtusa* Linn flowers, *Artabotrys hexapetalus* (Linn.f.) *Bhandari* stalks, *Nerium indicum* Mill leaves and *Oroxylum indicum* Vent leaves. The extract from *Nerium indicum* Mill leaves had highest β-glucosidase activity (26 nmole/min/g). The crude extract from plant tissues was also screened for β-glucosidase by incubating with arbutin in 0.1 M sodium acetate buffer pH 5.5 at 37^{o}C for overnight and analyzed by TLC. The results shown arbutin could not digested by the extract from 6 plant species, indicating that no activity of enzyme could be detected by arbutin (data not shown). In screening for β-glucosidase by its natural substrate, the crude extract was incubated with its crude substrate in 0.1 M sodium acetate buffer pH 5.5 at 37 ^{o}C for overnight and analyzed by TLC. The result shows the extract from the flowers of *P. obtusa* L. had hydrolytic activity toward its crude substrate as shown in (Figure 13) while β-glucosidase activity could not detected from other plant extracts by its natural substrate (data not shown).



Lane 1: Crude substrate from *P. obtusa* Linn flowers incubated with Thai Rosewood β-glucoside

Lane 2: Crude substrate from *P. obtusa* Linn flowers

Lane 3: Crude enzyme from *P. obtusa* Linn flowers

Lane 4: Crude substrate from *P. obtusa* Linn flowers incubated with crude enzyme from *P. obtusa* Linn flowers

Figure 13: Screening of β-glucosidase from *P. obtusa* Linn flowers by its natural substrate

The presence of β-glucosidase was performed by incubation the crude enzyme extracts from the flowers of *P. obtusa* Linn with its natural substrate. The band (crude natural substrate) in lane 2 was disappeared after digested with β-glucosidase from Thai rosewood and its crude enzyme extract (represent by block arrow, ⇨), and the new band with high mobility was appeared (dark arrow, ➔). Indicating that the crude extract from the flowers of *P. obtusa* Linn has a β-glucosidase activity towards its crude substrate.

Plant tissues	**Activity** (nmole/min/g)
Plumeria obtusa Lin	
Flowers	13
Stalks	0
Leaves	2
Tecoma stans Juss	
Flowers	0
Stalks	1
Leaves	1
Murraya paniculata Jack	
Stalks	0
Leaves	0
Nerium indicum Mill	
Stalks	0
Leaves	26
Oroxylum indicum Vent	
Stalks	0
Leaves	14
Artabotrys hexapetalus (Linn.f.)*Bhandar*i	
Flowers	0
Stalks	15
Leaves	0

Table 4: Screening of β-glucosidase from various plants by pNP-Glc (1 mM final concentration)

3.2 Partial purification of β-glucosidase and purification of a β-glucoside from the flowers of *Plumeria obtusa* Linn

3.2.1 Purification and structure elucidation of β-glucoside from the flowers of *Plumeria obtusa* Linn

β-glucoside from *P. obtusa* L. was purified by using Sephadex-LH20 gel filtration and preparative-TLC as described in section 2.3.1.2. The effluent containing β-glucoside was checked by TLC and presence of β-glucoside was confirmed by incubation with Thai Rosewood β-glucosidase. The β-glucoside could also be detected by its yellow colour. The pool of fractions containing β-glucoside was applied on a TLC plate and run with chloroform and methanol (3:1 v/v). The β-glucoside band was separated from TLC plate, dissolved in methanol. Silica gel was filtered to give the purified β-glucoside.

Structural elucidation of the compound was established by analyses of its spectroscopic data. The ^{1}H NMR spectrum (Appendix, Figure 1) of β-glucoside from the flowers of *P. obtusa* Linn are shown in Table 5. The spectra were identical to these of reported data (30). In addition, the chemical shift (δ) of 4.65 ppm and the coupling constant (J) of 8 Hz for the anomeric proton suggested a β-linkage at the anomeric carbon of glucose (31). The presence of a complicated pattern in the region δ 3.0-5.0, corresponded to the chemical shift of proton resonance in the sugar. The ^{13}C NMR spectrum is shown in Table 5 (Appendix, Figure 2). The data were similar to the ^{13}C NMR data of the known glucoside (30). The ^{1}H and ^{13}C NMR showed signals corresponding to a plumieride coumarate glucoside which has found in the bark of *Plumeria rubra* Linn. This data shows two glucosyl groups attached at C-1 and C-23. Analysis of 2D NMR (1H-1H COSY Appendix Figure 3, HMQC Appendix Figure 4, HMBC Appendix Figure 5) spectral data led to the complete structure of this compound as shown in Figure 14. Mass data (Appendix, Figure 6) of β-glucoside show the signal at m/z 801 corresponding to $[M+Na]^{+}$, hence a molecular weight of 778 was established. Enzymatic hydrolysis with purified β-glucosidase from the flowers of *Plumeria obtusa* Linn, gave glucose and aglycone. The structural elucidation of aglycone was established by NMR spectroscopic data. The ^{1}H and ^{13}C

NMR spectrum of aglycone are shown in Table 5 (Appendix, Figure 7,8 respectively). The structural data was similar to its β-glucoside, except that the glycosyl group was replaced by hydroxyl group at C-23. Analysis of 2D NMR (1H-1H COSY Appendix Figure 9, HMQC Appendix Figure 10, HMBC Appendix Figure 11) spectral data led to the complete structure of aglycone as shown in Figure 14. Mass data (Appendix, Figure 12) of aglycone showed the signal at m/z 639 corresponding to $[M+Na+H_2O]^+$, suggesting the molecular weight of aglycone is 598. This data suggested that one unit of glucose was removed at C-23.

Table 5: ^{1}H (400Mz) and ^{13}C (100 Hz) NMR spectral data of β-glucoside and aglycone from the flowers of *Plumeria obtusa* Linn

	β-glucoside		aglycone	
C	$\delta^{13}C$	$\delta^{1}H$, multiplicity ($J_{H,H}$/Hz)	$\delta^{13}C$	$\delta^{1}H$, multiplicity ($J_{H,H}$/Hz)
1	93.96	5.18, d (6.37)	92.97	5.28, d (5.6)
3	151.8	7.31, s	151.77	7.55, s
4	110.6	- -	109.55	- -
5	38.4	3.69, s	39.94	3.88, ddd
6	140.7	6.23, dd (5.46, 2.25)	141.21	6.43, dd (2.36, 2.36)
7	128.4	5.25, d (4.76)	129.03	5.53, dd (2.24, 2.33)
8	97.8	- -	96.74	- -
9	49.2	2.98, dd (3.14, 3.14)	49.81	2.84, dd (7.32, 5.76)
10	151.6	7.20, s	151.77	7.48, s
11	133.6	- -	133.44	- -
12	172.2	- -	170.17	- -
13	65.8	5.5 , d (6.48)	64.71	5.65, d (5.59)
14	19	1.41, d (6.51)	19.38	1.5, d (6.64)
15	168.8	- -	166.79	- -
16	52.4	3.58, s	51.42	3.67, s
17	167.7	- -	166.28	- -
18	115.8	6.15, d (15.99)	114.11	6.34, d (15.89)
19	146.1	7.42, d (15.85)	146.07	7.6, d (15.95)
20	128	- -	125.42	- -
21	130.6	7.28, d (8.53)	130.77	7.5, d (8.81)
22	117.3	6.93, d (8.62)	116.45	6.85, d (8.55)
23	159.2	- -	161.18	- -
24	117.3	6.93, d (8.62)	115.47	6.75, d (8.69)
25	130.6	7.28, d (8.53)	130.77	7.45, d (8.81)
glc-1	98.6	4.6 , d (7.92)	99.1	4.65, d (7.83)
glc-2	73.1	3.15, dd (8.41,8.67)	73.8	3.15, dd (8.03,8.58)
glc-3	76.0	3.47, dd (9.72,10.56)	77.1	3.35, dd (8.96)
glc-4	69.8	3.48, dd (9.72,10.56)	70.73	3.25, dd
glc-5	76.0	3.47, dd (9.72,10.56)	78.0	3.25, dd
glc-6	61.0	3.75, dd (11.67,12.59)	61.8	3.8, dd (11.42,11.42)
glc-1'	98.6	4.55, d (9.4)	-	-
glc-2'	73.3	3.1, dd (8.41,8.67)	-	-
glc-3'	76.5	3.42, dd (8.96,9.06)	-	-
glc-4'	70.0	3.42, dd (8.96,9.06)	-	-
glc-5'	76.5	3.42, dd (8.96,9.06)	-	-
glc-6'	61.2	3.7 , dd (11.67,12.59)	-	-

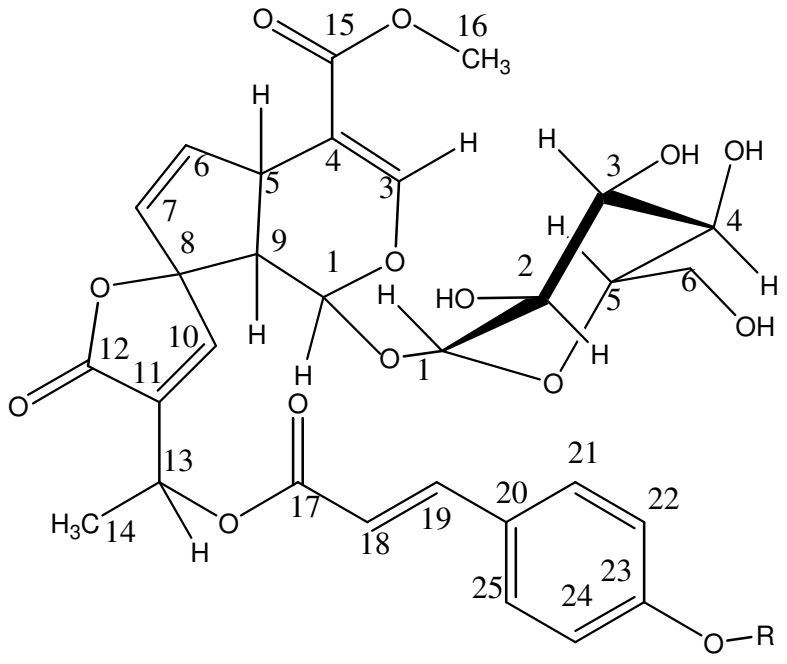


Figure 14: Structure of *Plumeria* β-glucoside

This compound contain 2 glycosyl groups attached at C-1 and C-23. After digestion with purified β-glucosidase from the flowers of *Plumeria obtusa* Linn, gave glucose and aglycone R= Glc, *Plumeria* β-glucoside

R= H , aglycone of *Plumeria* β-glucoside

3.2.2 Partial purification of β-glucosidase from the flowers *Plumeria obtusa* Linn.

β-glucosidase of *Plumeria obtusa* Linn was partially purified by 75% ammonium sulfate precipitation, DEAE-cellulose, Con A-Sepharose, Sepharose S-300 and Butyl-Sepharose chromatography, respectively. The summary of the partial purification of β-glucosidase is shown in Table 6. The brownish crude extract from the flowers of *Plumeria obtusa* Linn was fractionated by 0-75% saturation ammonium sulfate. Although the 0-75% fraction was dark brown, purification of β-glucosidase increased by 1.55 fold (from 8.11 nmole/min/mg to 12.6 nmole/min/mg), and 96 % yield was recovered.

The resuspended 0-75% ammonium sulfate was dialyzed with 10 mM phosphate buffer pH 8.0 and fractionated by DEAE- cellulose column chromatography, the elution profile (Figure 15) shown the contaminating protein was removed into the unbound and bound part. Fractions containing β-glucosidase activity was pooled from fraction no. 145-250. The pooled of fractions from DEAE-cellulose column chromatography was still brown. The purification fold was increased from the crude extract by 10.56 fold (from 8.11 nmole/min/mg to 85.63 nmole/min/mg). The pooled of fractions containing β-glucosidase were concentrated before loading onto a Con A-sepharose column chromatography. The elution profile (Figure 16) shown the β-glucosidase activity was found in bound part (from fraction no. 56-59), while most of contaminating protein was removed in the unbound part. Fractions 56-59 were pooled and used for further purification. The purification fold was increased from the crude extract to 18.2 fold (from 8.11 nmole/min/mg to 147.58 nmole/min/mg), and 12% yield was recovered. The pool of the Con A-sepharose column chromatography was concentrated to give 5% of column volume before loading onto the Sephacryl S-300 column. The chromatographic profile (Figure 17) shows that most of contaminating protein was removed in fractions 83-93, and β-glucosidase activity was found in fractions 66-80. The purification fold was increased from the crude extract to 45.6 fold (from 8.11 nmole/min/mg to 369.6 nmole/min/mg), and 10% yield was recovered. Fractions containing β-glucosidase activity were pooled and concentrated. $AmSO_4$ was added to the pool obtained from the Sephacryl S-300 column to a concentration of

1.5 M. The supernatant was then loaded onto a Butyl-Sepharose FF column. The elution profile (Figure 18) showed that the β-glucosidase activity was found in the bound part (from fraction no. 75-95), while most of the contaminating protein was removed in the unbound part (from fraction no. 2-10). The purification fold was increased from the crude extract to 143 fold (from 8.11 nmole/min/mg to 1160 nmole/min/mg), and 2.3% yield was recovered.

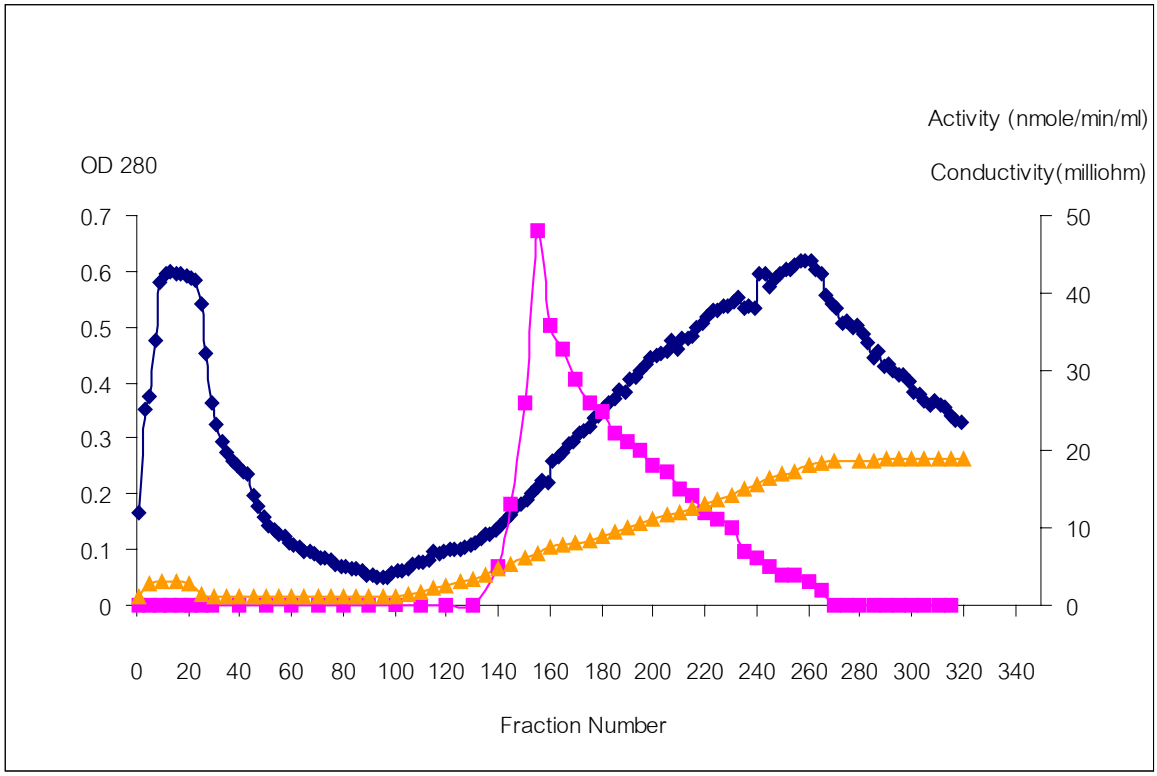
The purity of β-glucosidase was monitored by SDS-PAGE of the samples from some purification steps as describe in section 3.2.3. The cumulative yield and fold purification of the β-glucosidase, obtained from the last step of purification, were 2.3 % and 143 fold, respectively. The active fractions obtained from Sephacryl-S 300 were pooled and used for characterization of β-glucosidase.

Table 6: Summary of partial purification of β-glucosidase of *Plumeria obtusa* Linn

1 kg of flowers was used for purification. Assay of β-glucosidase was performed with 6 mM *Plumeria* β-glucoside at pH 5.5 and 40°C.

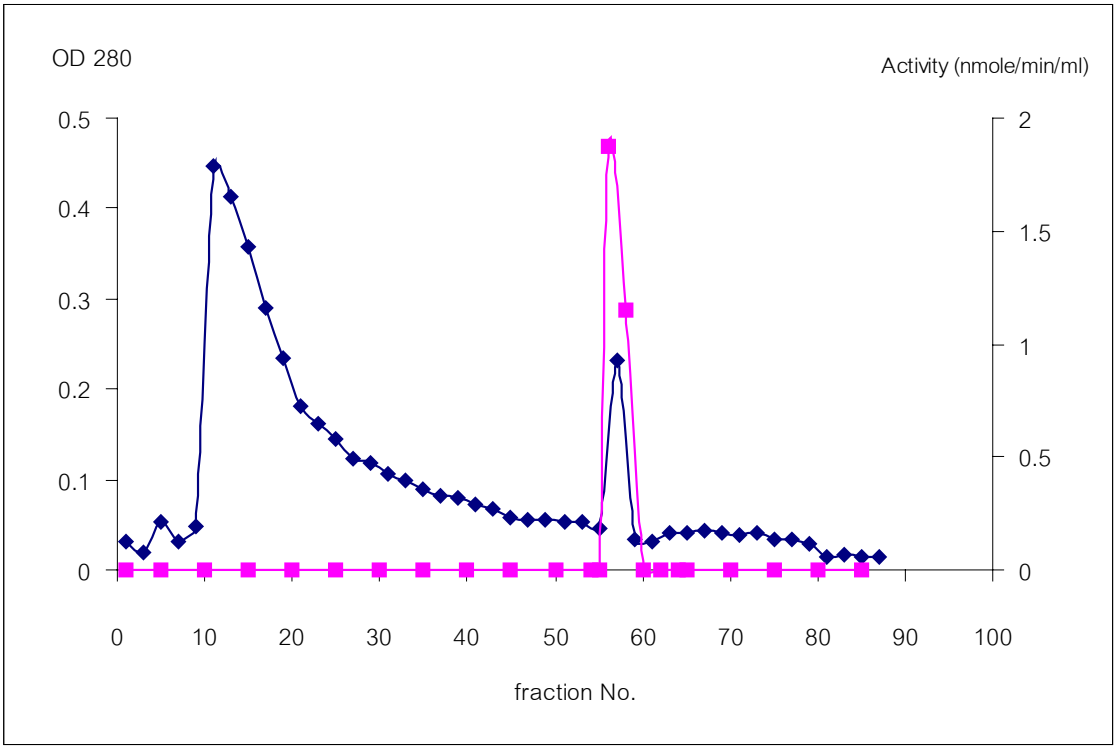
Purification step	Total activity (nmole/min)	Total protein (mg)	Specific activity (nmole/min/mg)	Yield (%)	Purification (fold)
Crude extract	5001	975	8.11	100	1
75%$(NH_4)_2$ SO_4	4833	383.6	12.6	96	1.6
DEAE-cellulose	1651	19.3	85.6	33	10.6
Con A-Sepharose	580	3.9	147.6	12	18.2
Sephacryl S-300 HR	510	1.4	369.6	10	45.6
Butyl-Sepharose FF	116	0.1	1160	2.3	143

Figure 15: Chromatographic profile of DEAE-Cellulose fractionation from the 75% $AmSO_4$ fraction



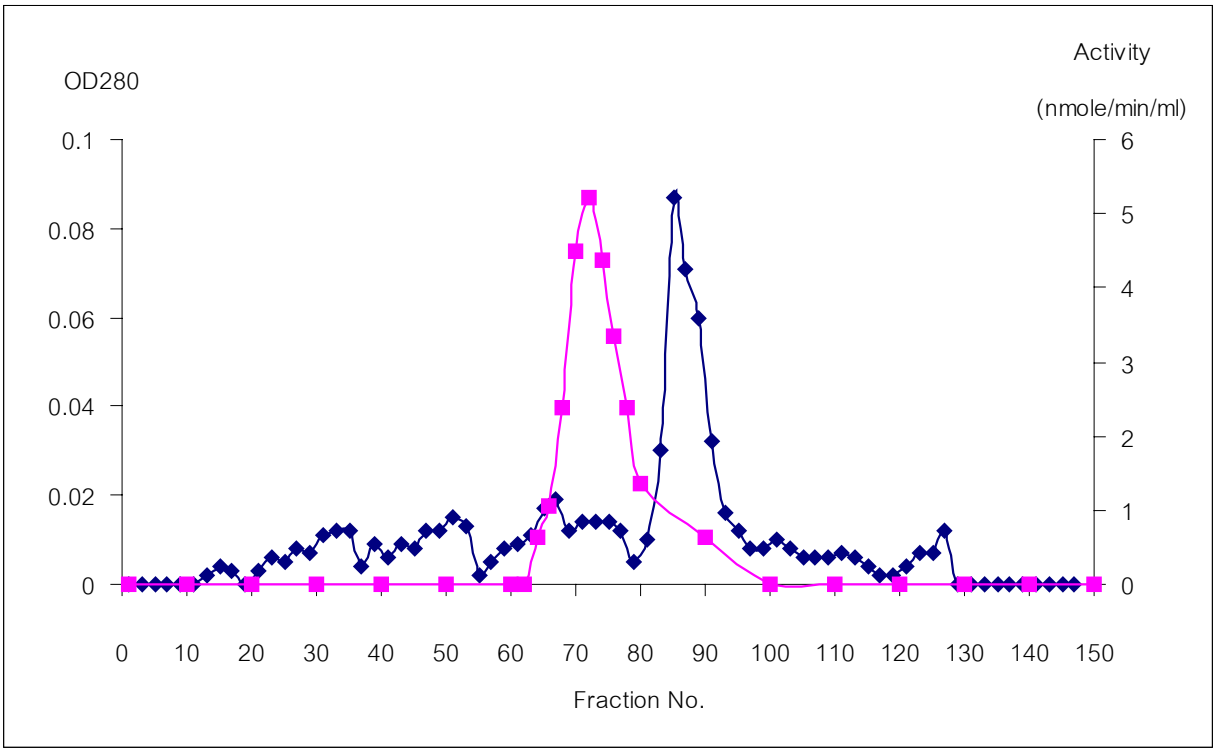
The dialyzed sample containing 383.6 mg protein was applied on DEAE-cellulose equilibrated with 10 mM sodium phosphate buffer pH 8.0. The column was eluted with a linear gradient of 0-0.3 M NaCl at the flow rate of 0.5 ml/min. Fractions of 3 ml were collected and assayed for β-glucosidase (—■—), protein concentration (OD 280 , —◆—) and conductivity (—▲—).

Figure 16: Con A –Sepharose chromatography of β-glucosidase obtained from the DEAE-Cellulose column



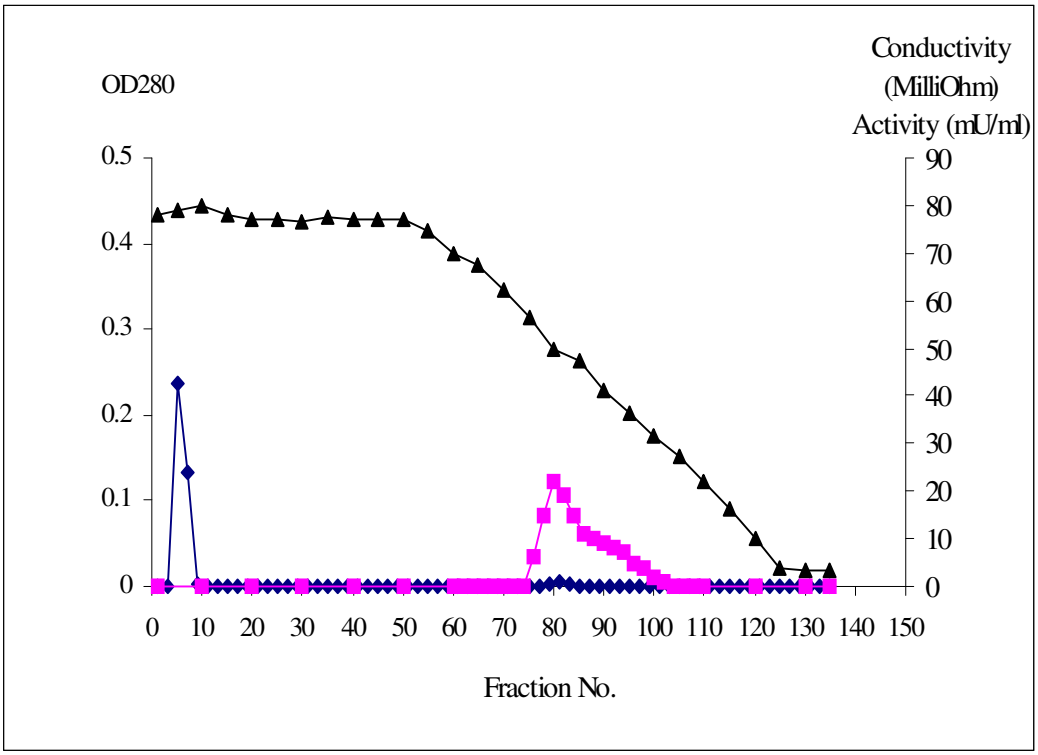
The enzyme, containing 19.28 mg protein, was loaded to the Con A-Sepharose column equilibrated with 20 mM sodium phosphate pH 7.0 containing 0.5 M NaCl. The column was eluted by 0.3 M methyl-α-mannoside at the flow rate of 0.5 ml/min. Fractions of 3 ml were collected and used for measurement of β-glucosidase (—■—) and protein concentration (OD 280, —◆—).

Figure 17: Sephacryl S-300 HR filtration of β-glucosidase obtained from the Con A-Sepharose column.



The β-glucosidase pool containing 3.93 mg protein obtained from Con A-sepharose column was concentrated and loaded onto Sephacryl S-300 column equilibrated in 0.1 M sodium acetate buffer pH 5.5 containing 0.15 M NaCl. The column was eluted with the same buffer at a flow rate of 0.5 ml/min. Fractions of 3 ml were collected and determined for β-glucosidase (—■—)as well as for protein content (OD 280, —◆—).

Figure 18: Butyl-Sepharose chromatography of β-glucosidase obtained from Sephacryl S-300 filtration.

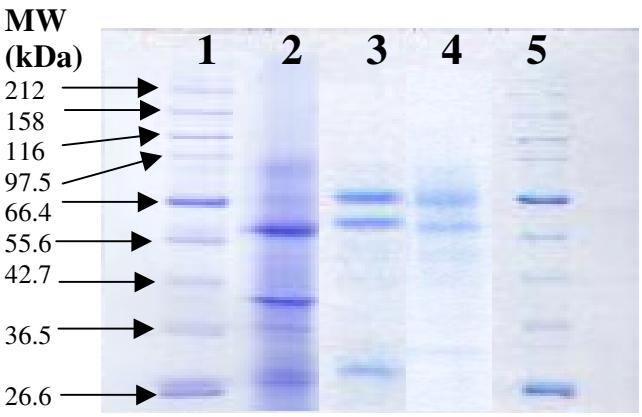


The enzyme contain 1.38 mg protein was loaded to the Butyl-sepharose FF column chromatography equilibrated with 1.5 M $(NH_4)_2SO_4$ in 0.05 M sodium phosphate buffer pH 7.0. The column was washed with the same buffer followed by a linear gradient of 1.5 – 0 M $(NH_4)_2SO_4$ at the flow rate of 0.5ml/min. Fractions of 3 ml were collected and used for measurement of β-glucosidase (—■—), protein concentration (OD 280, —◆—) and conductivity (—▲—).

3.2.3 Determination of purity of β-glucosidase from the flowers of *Plumeria obtusa* Linn. by SDS-PAGE

β-glucosidase from the flowers of *Plumeria obtusa* Linn was analyzed by SDS-PAGE in a 10% gel (Figure 19). The mobility of protein bands was compared to that of marker protein as shown in Figure 20. Denatured β-glucosidase gave 2 bands of 59 kDa and 67 kDa (lane 4, Figure 19). In comparison, the pool of Sephacryl S-300 in lane 3 contains 3 bands of 59 kDa 67 kDa and 30.14 kDa. Compared to the crude extract proteins (lane 2, Figure 19), β-glucosidase has been purified to a significant degree.

Figure 19: SDS-PAGE of step of purification of β-glucosidase from *Plumeria obtusa* Linn.



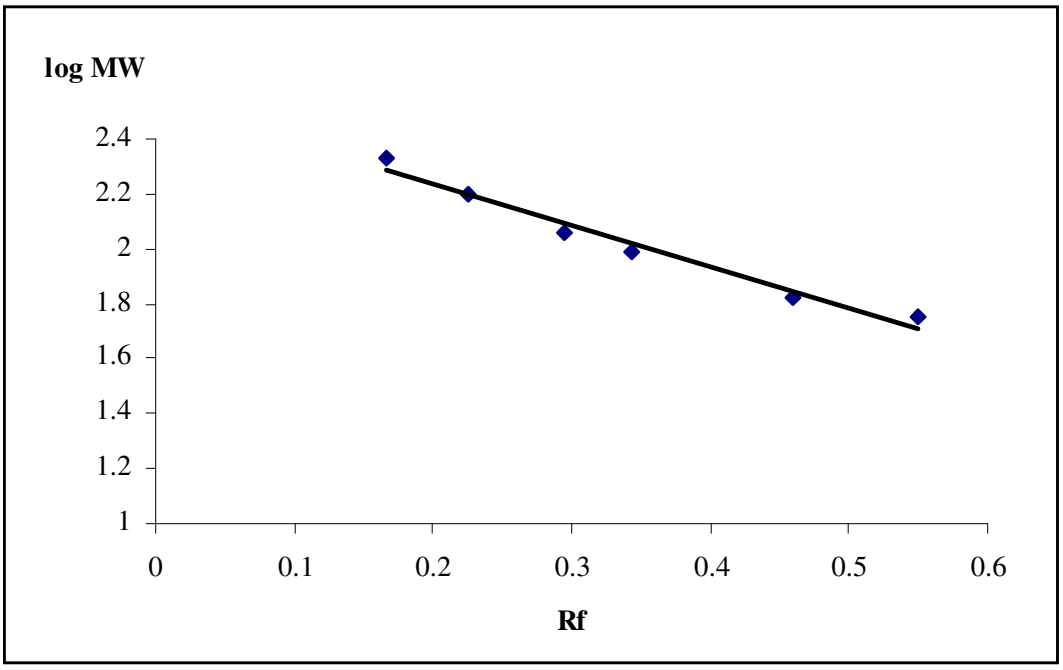
Lane 1,5: Standard molecular weight marker

Lane 2: Crude extract

Lane 3: Fractions of Sephacryl S-300 column

Lane 4: The bound fractions of Butyl-Sephasore column

Figure 20: Standard calibration curve of β-glucosidase from the flowers of *Plumeria obtusa* Linn on SDS-PAGE.

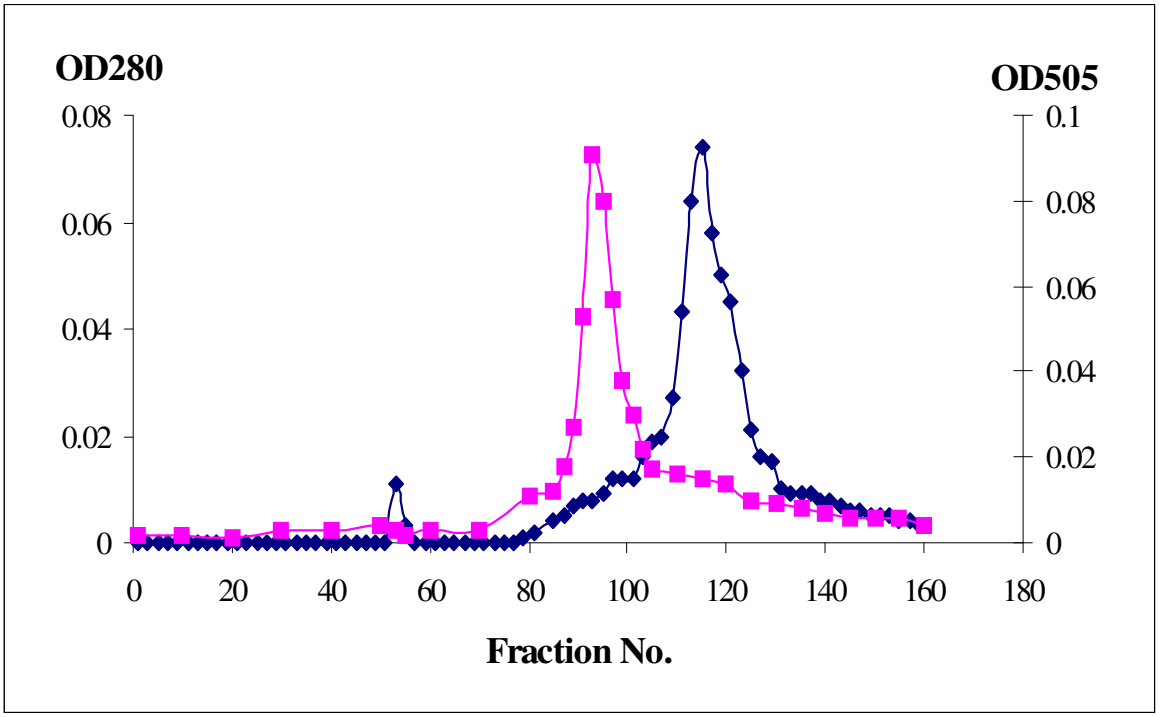


3.3 Characterization of β-glucosidase from the flowers of *Plumeria Obtusa* Linn

3.3.1 Determination of native molecular weight of β-glucosidase

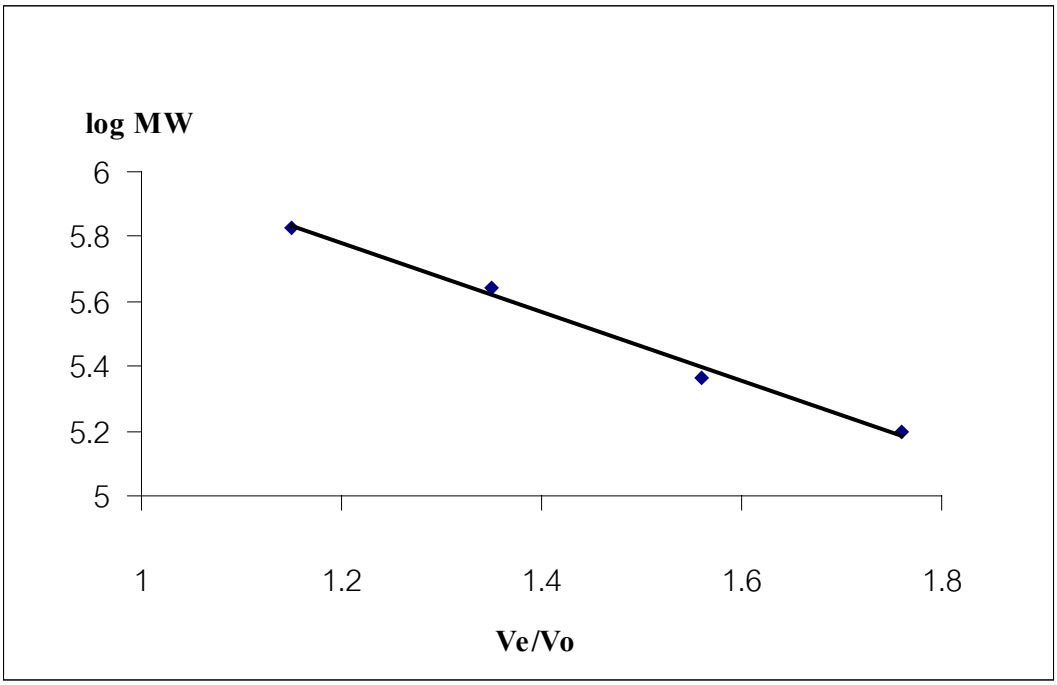
To determine the native molecular weight of β-glucosidase, fractions of Con A-sepharose was applied on Sephacryl S-300 column. The elution profile was shown in Figure 21. The column was calibrated with various standard proteins (thyroglobulin, ferritin, catalase and aldolase). The Ve/Vo of each standard marker was plotted against log molecular weight of the protein (Figure 22). The native molecular weight of β-glucosidase was estimated to be in a range of 181,000 $\pm$ 1,000 Da.

Figure 21: Sephacryl S-300 filtration of β-glucosidase



The β-glucosidase obtained from Con A-sepharose column was concentrated and loaded onto Sephacryl S-300 column equilibrated in 0.1 M sodium acetate buffer pH 5.5 containing 0.15 M NaCl. The column was eluted with the same buffer at a flow rate of 0.5 ml/min. Fractions of 3 ml were collected and determined for β-glucosidase (OD505,–■–)as well as for protein content (OD 280, –◆–).

Figure 22: Standard calibration curve of Sephacryl S-300

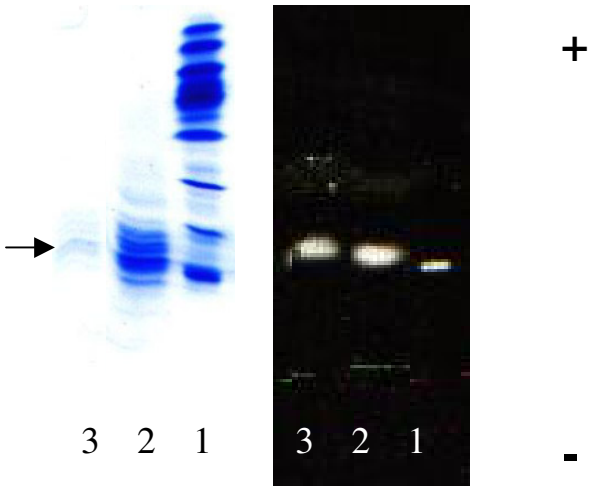


The column (400 ml of volume column) was calibrated with the following standard proteins: thyroglobulin (669,000 Da), ferritin (440,000 Da), catalase (232,000 Da) and aldolase (158,000 Da). Blue dextran (2,000,000 Da) was used to determine void volume.

3.3.2 Agarose isoelectric focusing

To study the heterogeneity of the β-glucosidase and to determine pI of the enzyme, agarose isoelectric focusing was used. The sample was applied and run on an agarose IEF gel with pH gradient of 3.5-10 and stained with Coomassie Blue R-250 and 4-MU-Glc. (Figure 23) One fluorescent band of β-glucosidase was found with pI about 5.0.

Figure 23: Agarose isoelectric focusing (IEF) gel stained for β-glucosidase activity.



Lane 1: Standard marker for IEF pI 4.45-9.6, the fluorescent band corresponding to phycocyanin with pI 4.75

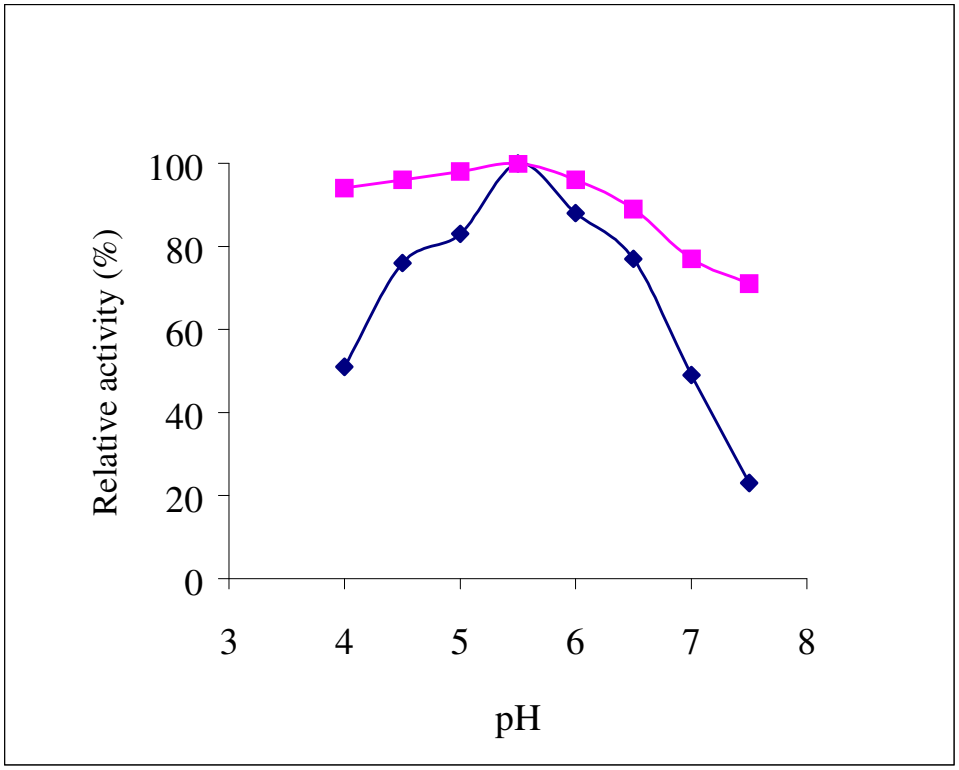
Lane 2: Fractions from Sephacryl S-300 column

Lane 3: Fractions from Butyl-Sepharose column

3.3.3 pH optima of β-glucosidase from the flowers of *Plumeria obtusa* Linn

The activity of purified β-glucosidase was assayed at 40 °C in 0.1 M McIlvaine buffer at pH 4.0,4.5,5.0,5.5,6.0,6.5,7.0 and 7.5. The results showed that the maximum activity of β-glucosidase towards both pNP-Glc and its natural substrate were obtained at pH 5.5 (Figure 24).

Figure 24: pH optima of β-glucosidase from *Plumeria obtusa* Linn towards pNP-Glc and its natural substrate.



100% was corresponding to 2930 nmole/min/mg when pNP-Glc was used as substrate (—◆—). When the β-glucoside from the flowers of *Plumeria obtusa* Linn was used as substrate (—■—), 100% was corresponding to 307 nmole/min/mg

3.3.4 Kinetic studies of β-glucosidase from the flowers of *Plumeria Obtusa* Linn

3.3.4.1 With pNP-Glc

Fractions from Sephacryl S-300 were assayed at pH 5.5, 40°C for various times from 10-60 min. From the results (Figure 25), shown that the reaction continued linearly for about 10-60 min. For kinetic studies of the enzyme, a time of 30 min was selected. The enzyme was assayed at pH 5.5, 40 °C using various concentration of pNP-Glc (0.2-20 mM) in 0.1 M sodium acetate buffer for 30 min. The Michaelis-Menten curve for β-glucosidase was shown in Figure 26. The K_m of β-glucosidase calculated by non-linear regression was 2.79 ± 0.1 mM while the V_{max} was 0.751 ± 0.01 μmole/min/mg (Table 7).

Figure 25: Determination of suitable reaction time of β-glucosidase with pNP-Glc as substrate

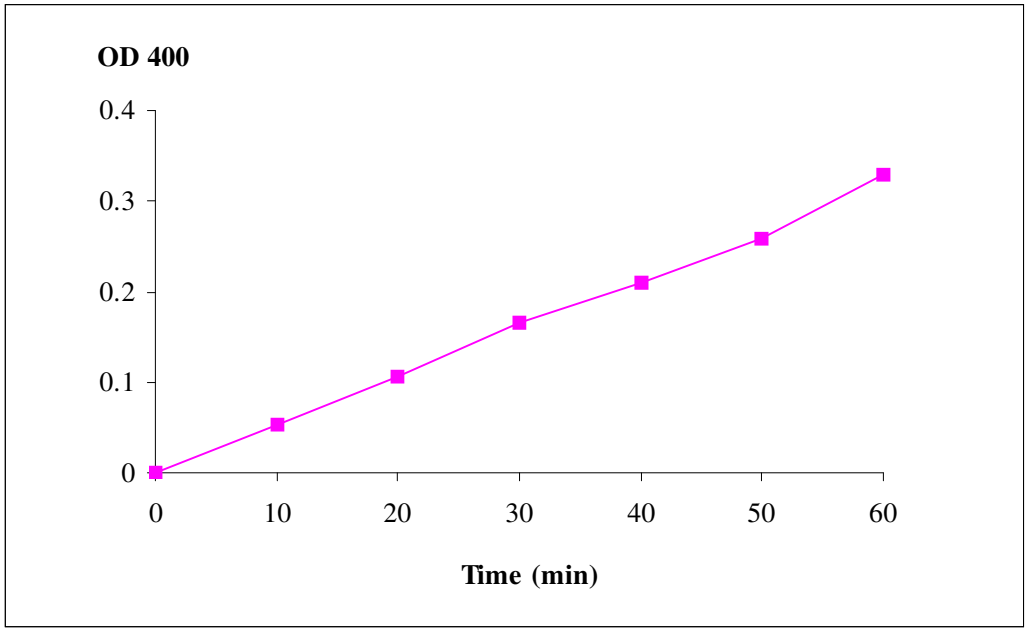
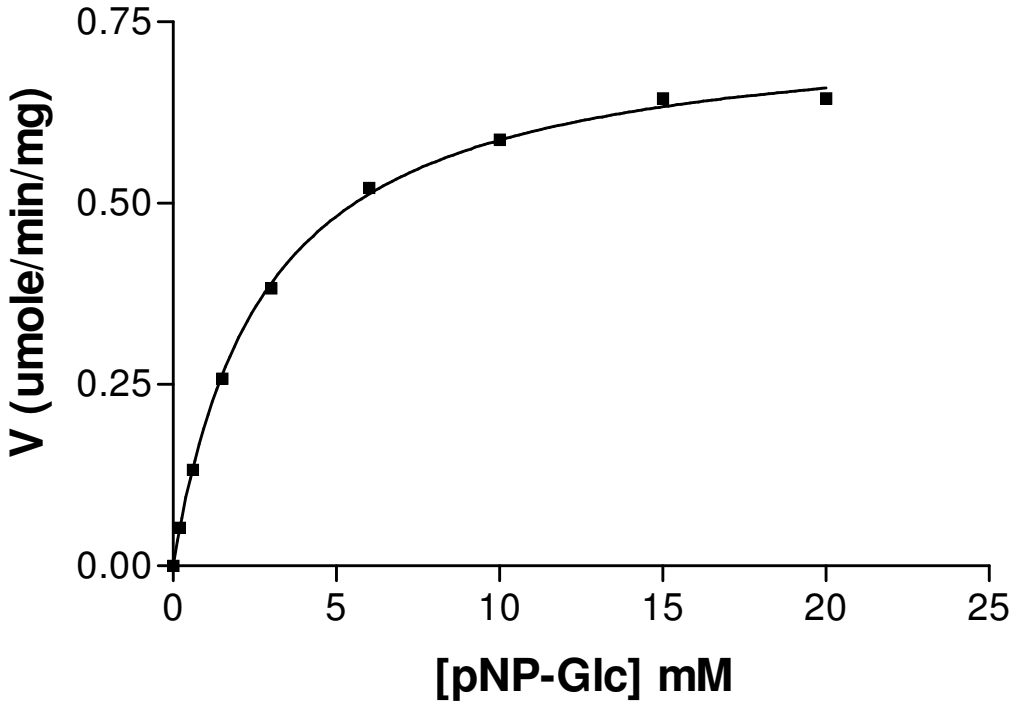


Figure 26: Michaelis-Menten plots of β-glucosidase with pNP-Glc as substrate



Michalis-Menten was plotted using the prism 3 program (Graphpad software)

3.3.4.2 With β-glucoside from the flowers of *Plumeria obtusa* Linn as substrate

β-glucosidase from Sephacryl S-300 was assayed at pH 5.5, 40°C at various times from 5-60 min. The results (Figure 27), showed that the reaction continued linearly for about 5-30 min. For kinetic studies of the enzyme, a time of 30 min was selected. The enzyme was assayed at pH 5.5, 40 °C using various concentration of β-glucoside from *Plumeria obtusa* Linn (0.1-6 mM) in 0.1 M sodium acetate buffer for 30 min. The Michaelis-Menten curve for β-glucosidase was shown in Figure 28. The K_m of β-glucosidase for its natural substrate, calculated by non-linear regression, was 0.33 ± 0.02 mM while the V_{max} was 0.23 ± 0.003 μmole/min/mg (Table 7).

Figure 27: Determination of suitable reaction time of β-glucosidase with natural substrate

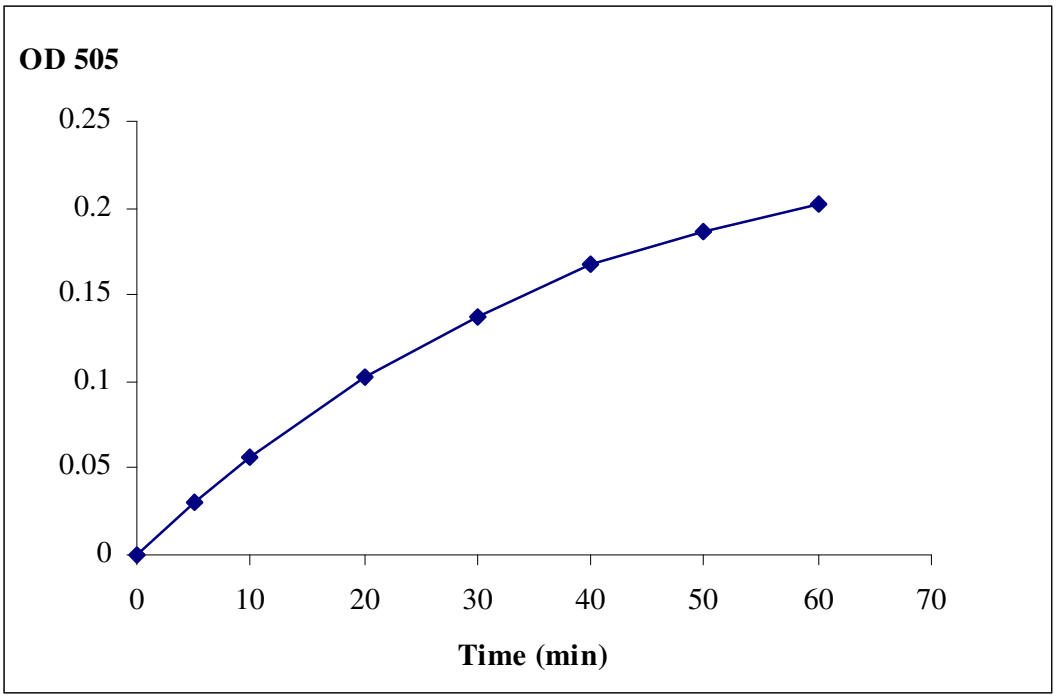
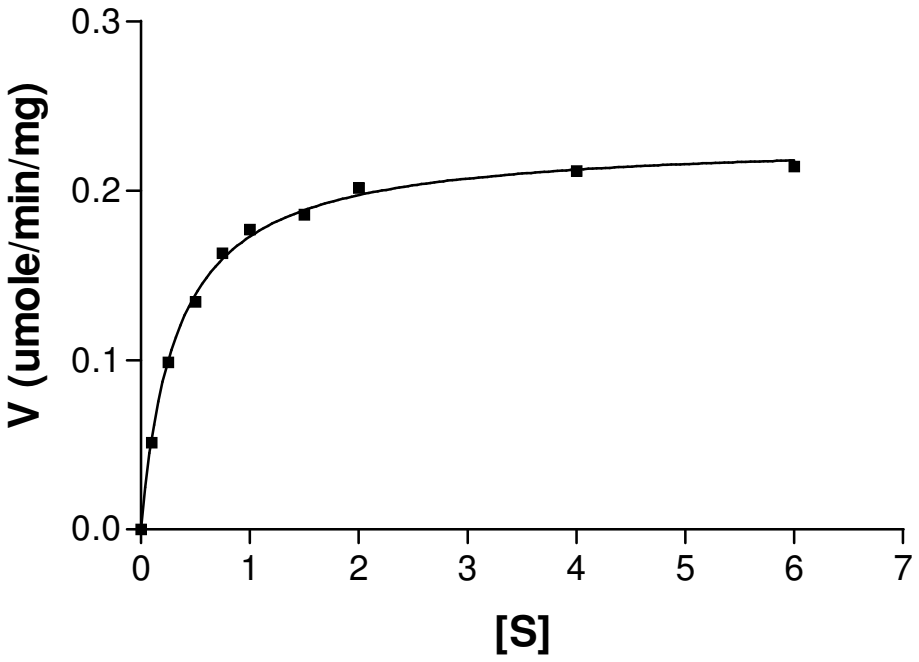


Figure 28: Michaelis-Menten plots of β-glucosidase with natural substrate



Michalis-Menten was plotted using the prism 3 program (Graphpad software)

Table 7: Kinetic properties of β-glucosidase from *Plumeria Obtusa* Linn

Substrate	**V_{max} (μmole/min/mg)**	**K_m (mM)**
Plumeria β-glucoside	0.23 ± 0.003	0.33 ± 0.02
PNP-Glc	0.75 ± 0.01	2.79 ± 0.1

3.3.5 Studies on hydrolytic specificity of β-glucosidase

β-glucosidase activity using different pNP-glycosides was compared at a final concentration of 5 mM, pH 5.5 at 40^{o}C. The enzyme had hydrolytic activity towards pNP-β-D-Glc, pNP-β-D-Fuc, pNP-β-D-Gal and pNP-β-D-Man, and had low activity (less than 10 %) for other pNP-glycosides as shown in Table 8. With natural substrates (Table 9), enzyme had lower activity towards dalcochinin-β-glucoside (natural substrate of Thai Rosewood β-glucosidase) compared to its natural substrate, and had no activity towards cyanogenic glucoside (prunasin, linamarin and amygdalin), natural substrate of *Solanum torvum* (torvoside H), disaccharides (gentiobiose), aromatic glucosides (arbutin and salicin), alkyl glucosides (methyl-glucosides and hexyl-glucosides).

Table 8: Hydrolytic of pNP-glycosides by the purified β-glucosidase

Substrates	**% Relative activity**
pNP-β-D-glucoside	100
pNP-β-D-fucoside	131
pNP-β-D-mannoside	29
pNP-β-D-galactoside	36
pNP-α-D-glucoside	4
pNP-α-L-fucoside	8

100% was corresponding to 2377 nmole/min/mg when pNP-Glc was used as substrate

Table 9: Hydrolytic of natural substrate and alkyl β-glucosides by the purified β-glucosidase compared with its natural substrate.

Substrates	% Relative activity
β-glucoside from *P. obtusa* L.	100
Dalcochinin-β-glucoside	4.02
Torvoside H	0
Prunasin	0
Linamarin	0
Amygdalin	0
Esculin	3.08
Salicin	0
Arbutin	0
Gentiobiose	0
Methyl-β-D-glucoside	0
Hexyl-β-D-glucoside	0
pNP-β-glucoside	105.4
4-MU-glucoside	30.88

100% activity corresponded to 740 nmole/min/mg when β-glucoside from *P. obtusa* L. was used as substrate.

CHAPTER IV
DISCUSSION

β-glucosidases are useful for many applications, such as synthesis of aryl or alkyl glucosides. So novel β-glucosidases were screened for from Thai plants. First, the β-glucosides were screened for by incubating the crude extract of the plant with Thai rosewood, almond or cassava-stem β-glucosidase, because these 3 enzymes have different substrate specificity and different functions (32). Our findings agree with unpublished data of Boonpuan, who showed that the crude extract of *Plumeria* spp., *Nerium indicum* Mill and *Murraya paniculata* Jack showed high content of β-glucosides. So, these plants may be have a β-glucosidase with specificity to its natural substrate. To confirm the presence of β-glucosidase, the crude extract of plant was incubated with synthetic substrate (pNP-Glc, arbutin) or its natural substrate. The extract from the flowers of *Plumeria obtusa* Linn showed hydrolytic activity toward pNP-Glc and its crude natural substrate. This shows that the flower of *P. obtusa* L is a source of both β-glucoside and β-glucosidase. The natural substrate of β-glucosidase from the flowers of *P. obtusa* L was isolated and identified for use to assay the enzyme activity. Structure elucidation of a natural substrate of β-glucosidase was established by NMR spectroscopy and comparison of its analogues (30). This suggested the structure plumieride coumarate, having two glycosyl groups attached. The position of the glycosyl group was shown to be at C-23 by the ca 2 ppm downfield shift of C-23 in the ^{13}C spectrum. The structure of β-glucoside was confirmed by mass spectroscopic data. Mass data of β-glucoside from the flowers of *P. obtusa* L showed the signal at 801 m/z corresponding to $[M+ Na]^+$, suggesting that the molecular weight of the β-glucoside should be 778. It is a known iridoid glucoside, namely “Plumieride coumarate glucoside”. Previously, found in the bark of *P. rubra* L collected in Indonesia (33). The natural substrate of β-glucosidase isolated from the flowers of *P. obtusa* L is well hydrolyzed by other β-glucosidases (Thai rosewood, almond or cassava-stem β-glucosidase). The iridoid glucoside was incubated with

purified β-glucosidase from the flowers of *P. obtusa* L. to give the aglycone and D-glucose (analyzed by TLC). Structural elucidation of the aglycone was established by analyses of their spectroscopic data in by comparison to those published previously in the literature (30). Mass data of the aglycone showed a signal at m/z 639 corresponding to $[M+Na+H_2O]^+$, giving a molecular weight of 598. This spectral data suggested that one unit of glucose was removed after digestion with purified β-glucosidase from the flowers of *P. obtusa* L. The ^1H, ^{13}C NMR spectra of aglycone were less complex than those of the glucoside, particularly in the sugar resonance. An analysis of DEPT, 1H-1H COSY, HMQC and HMBC suggested that the hydrolysis is occur at C-23 position.

Many β-glucosides are known to be active in the presence of their specific β-glucosidase. This means that the β-glucosides (aglycone or a related intermediate) is responsible for the biological activity. Iridoids are known to have a variety of biological effects and have been implicated to play roles in plant-herbivore and pray-predator interaction (34,35). Some iridoids have shown to be a plant toxins (36,37), and could be activated into alkylating agents by β-glucosidase. These structures readily bind to various biological nucleophiles, including nucleophilic side chains of proteins, and therefore exert adverse effects on herbivores (e.g., loss of nutritive value caused by loss of Lys and inactivation of enzymes), providing the plants with a chemical defense against herbivores (38-42). Some iridoids, such as plumieride have also been reported as a plant growth inhibitors on wheat seedling. However, the chemical or physiological bases for the biological activity of iridoid glucosides are still unclear. Almost all higher plants contain secondary metabolic products. β-glucoside and aglycone could be detected in other tissues of *P. obtusa* L, such as leaf and stem, but the levels of β-glucosidase in these other tissues is lower than the level found in the flowers. The high levels of both enzyme and natural substrate in the flowers suggest that both play important biological roles in the flower. These data suggested that β-glucoside from the flowers of *P. obtusa* L and its specific β-glucosidase may function together, for example in the storage of glucose or as a defence against insects or herbivores.

The flowers of *P. obtusa* L are a source for the purification of β-glucosidase, because of their low content of chlorophyll. However, PVPP and Dowex was added during purification to eliminate phenolic compounds. PMSF an inhibitor for serine protease, was also added. When the concentration of $AmSO_4$ reached 75% saturation, most of β-glucosidase was precipitated, and undesired protein, phenolic compounds, other pigments and small metabolites were removed into the supernatant. The next step of purification is DEAE-cellulose column chromatography because of its high capacity (0.68 g/ g of dry weight). Fractions from DEAE-cellulose column were dark brown corresponding to the OD_{280} peak, suggesting that phenolic compounds and pigments could interfere with the absorbance at 280 nm. Purification of β-glucosidase increased by 10.56 fold in this step. Con A-Sepharose column chromatography was used because of it was useful for removing other proteins, especially the unglycosylated protein. The elution profile of Con A-Sepharose column showed high efficiency to separate protein in the unbound part, from the bound activity peak of β-glucosidase, which corresponded to the protein peak. At this step, purification fold was 18.20. After that, Sephacryl S-300 column chromatography was used to remove the low molecular weight proteins. The elution profile of Sephacryl S-300 showed high efficiency to separate other protein, since fractions with no β-glucosidase activity but high protein concentration were clearly separated from fractions containing β-glucosidase activity. Additionally, some pigments were removed by this column. Purification of β-glucosidase at this step increased by 45.57 fold. The last step of purification was Butyl-Sepharose column chromatography. When the concentration of $AmSO_4$ was 1.5 M, some proteins could not bind to the column. The β-glucosidase was still bound and could be eluted with a linear gradient of 1.5 – 0 M $AmSO_4$. The elution profile of the Butyl-Sepharose column showed high efficiency to separate other protein, and the cumulative purification was also increased to 143 fold.

The β-glucosidase obtained from the last step of purification was purified to a significant degree, and showed 2.3 %yield and 143 fold purification. The purified enzyme shows two major bands at 59 and 67 kDa on SDS-PAGE similar to the subunit molecular weight of most β-glucosidase previously reported (55-65 kDa). It is possible that the purified β-glucosidase from this plant exists as a heterodimeric

enzyme, consisting of two different types of subunits of M_r 59 and/or 67 kDa. However, the band of 58.62 kDa may conceivably be a contaminating protein or degradation product of the band of 67 kDa. Rough molecular weight estimates by gel filtration show the native molecular weight of enzyme was 180 kDa. The natural abundance of trimeric enzyme is very rare, but a review of the literature (43) showed β-glucosidase from *Aspergillus niger* have subunit molecular weight of 110 kDa while the native molecualr weight is 330 kDa. These data suggesting that the purified β-glucosidase from the flowers of *P. obtusa* L appears to oligomeric enzyme which may be have 2-4 subunits. Purified β-glucosidase had a pI of 5.0 by agarose isoelectric focusing similar to those of other common β-glucosidase. These data suggested that the purified β-glucosidase from the flowers of *P. obtusa* L was a acidic oligomeric glycoprotein.

Optimum activity of β-glucosidase, when pNP-Glc and β-glucoside from the flowers of *P. obtusa* L were used as substrate, was observed at pH 5.5 which similar to those of β-glucosidase from Thai rosewood (12), soybean (44), ginseng (45) and valencia oranges (46). Kinetic parameters of β-glucosidase were determined for its natural substrate compared to a synthetic substrate (pNP-Glc). Km values for its natural substrate (0.33 mM) was lower than for synthetic substrate (2.9 mM). These results suggested that β-glucosidase has a higher affinity for its natural substrate than for the synthetic substrate. Enzymatic activity studies were performed in order to test substrate specificity for the glycone and aglycone moiety. Purified β-glucosidase from the flowers of *P. obtusa* L shows hydrolytic activity to both pNP-β-D-Glc and pNP-β-D-Fuc, but had lower activity for other pNP-glycosides (pNP-β-D-Man, pNP-β-D-Gal, pNP-α-D-Glc, pNP-α-L-Fuc). The enzyme with both β-glucosidase and β-fucosidase activities was also identified from seeds of Thai rosewood by Srisomsap *et al* (12). These results suggest that the purified enzyme showed a broad substrate specificity for the glycone moiety.

When the hydrolysis activity of β-glucosidase toward natural substrate, alkyl-glucosides and some synthetic substrate was tested to study aglycone specificity, β-glucosidase was able to hydrolyze dalcochinin β-glucoside (natural substrate of Thai rosewood β-glucoside) and esculin, but does not hydrolyze cyanogenic glucoside

(prunasin, linamarin and amygdalin), natural substrate of *Solanum torvum* (torvoside H), disaccharides (gentiobiose), aromatic glucosides (arbutin and salicin), alkyl glucosides (methyl-glucosides and hexyl-glucosides). Our data shows that most β-D-glucosides with an aromatic aglycone can be substrates for the enzyme, except for arbutin and salicin, which are not hydrolyzed. These results showed a narrow substrate specificity for the aglycone moiety. It possible that purified β-glucosidase from the flowers of *P.obtusa* L has a large hydrophobic site for aglycone binding. Many studies on substrate specificity have been performed for β-glucosidases from widely different sources, and several β-glucosidases are known to hydrolyze β-D-fucoside, β-D-galactoside, β-D-mannoside and steroid glucoside. Our finding shows a novel β-glucosidase with high specificity towards iridoid glucoside (Plumieride coumarate glucoside).

The properties of purified β-glucosidase from the flowers of *P.obtusa* L were compared to other β-glucosidases (Thai rosewood, almond, cassava-stem, soybean, ginseng, valencia oranges and vanilla beans β-glucosidase). Some properties (pH optimun of hydrolysis, pI and subunit molecular weight) of the enzyme shows similarity to β-glucosidases from Thai rosewood, valencia oranges, ginseng and *Polygonum tinctorium*, which classified in family1. Thus the purified β-glucosidase from the flowers of *P. obtusa* L seems to be a new member of the family 1 β-glucosidase.

CHAPTER V
CONCLUSION

1. β-glucoside was found in the extracts of *Plumeria obtusa* Linn, *Plumeria rubra* Linn, *Murraya paniculata* Jack, *Nerium indicum* Mill, *Oroxylum indicum* Vent, *Tecoma stans* Juss, *Artabotrys hexapetalus* (Linn.f.) *Bhandari and Rhinacanthus nasutus* KurZ. The extracts containing β-glucoside from *Plumeria obtusa* Linn, *Plumeria rubra* Linn were well digested by Thai rosewood, almond and cassava-stem β-glucosidase while the extracts from *M. paniculata* J, *N. indicum* M, *O. indicum* V, *A. hexapetalus* (L.) *Bhandari* and *R. nasutus* K were well digested by Thai rosewood and almond β-glucosidase, but poorly digested by cassava-stem β-glucosidase. The extracts from *T. stans* J were well digested by almond β-glucosidase but poorly digested by other β-glucosidase (Thai rosewood, cassava-stem β-glucosidase).
2. β-glucosidase activity was detected from the flowers of *P. obtusa* L., stalks of *A. hexapetalus* (L.) *Bhandari* , leaves of *N. indicum* M and leaves of *O. indicum* V by pNP-Glc. In addition, the extracts from the flowers of *P. obtusa* L shows hydrolytic activity towards its crude substrate.
3. The natural substrate from the flowers of *P. obtusa* L was isolated and identified. It is a known iridoid glucoside contain two glucosyl groups attached at C-1 and C-23. After digestion with its specific β-glucosidase gave β-D glucose and aglycone. Structure elucidation of aglycone by NMR spectroscopy shows hydrolysis is occurs at C-23 of the compound.
4. β-glucosidase was purified from the flowers of *P. obtusa* L by DEAE-cellulose, Con A-Sepharose, Sephacryl S-300 and Butyl-Sepharose. The properties of purified enzyme was also studied.
    4.1 The purified β-glucosidase gave 2 bands of 67 kDA and 59 kDa on SDS-PAGE.

4.2 Native molecular weight of the purified enzyme was estimate to be 181,000 $\pm$ 1000 Da by gel filtration (Sephacryl S-300 column chromatography).

4.3 The purified β-glucosidase have pI about 5.0 by agarose isoelectric focusing.

4.4 The maximum activity of β-glucosidase towards both pNP-Glc and its natural substrate were obtained at pH 5.5.

4.5 K_m of β-glucosidase for pNP-Glc, calculated by non-linear regression, was 2.79 $\pm$ 0.1 mM while the V_{max} was 0.751 $\pm$ 0.01 μmole/min/mg. The K_m of β-glucosidase for its natural substrate, calculated by non-linear regression, was 0.33 $\pm$ 0.02 mM while the V_{max} was 0.23 $\pm$ 0.003 μmole/min/mg.

4.6 The purified enzyme had hydrolytic activity towards pNP-β-D-Glc, pNP-β-D-Fuc, pNP-β-D-Gal and pNP-β-D-Man, and had low activity (less than 10 %) for other pNP-glycosides. The purified β-glucosidase had lower activity towards dalcochinin-β-glucoside (natural substrate of Thai Rosewood β-glucosidase) compared to its natural substrate, and had no activity towards cyanogenic glucoside (prunasin, linamarin and amygdalin), natural substrate of *Solanum torvum* (torvoside H), disaccharides (gentiobiose), aromatic glucosides (arbutin and salicin), alkyl glucosides (methyl-glucosides and hexyl-glucosides).

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APPENDIX

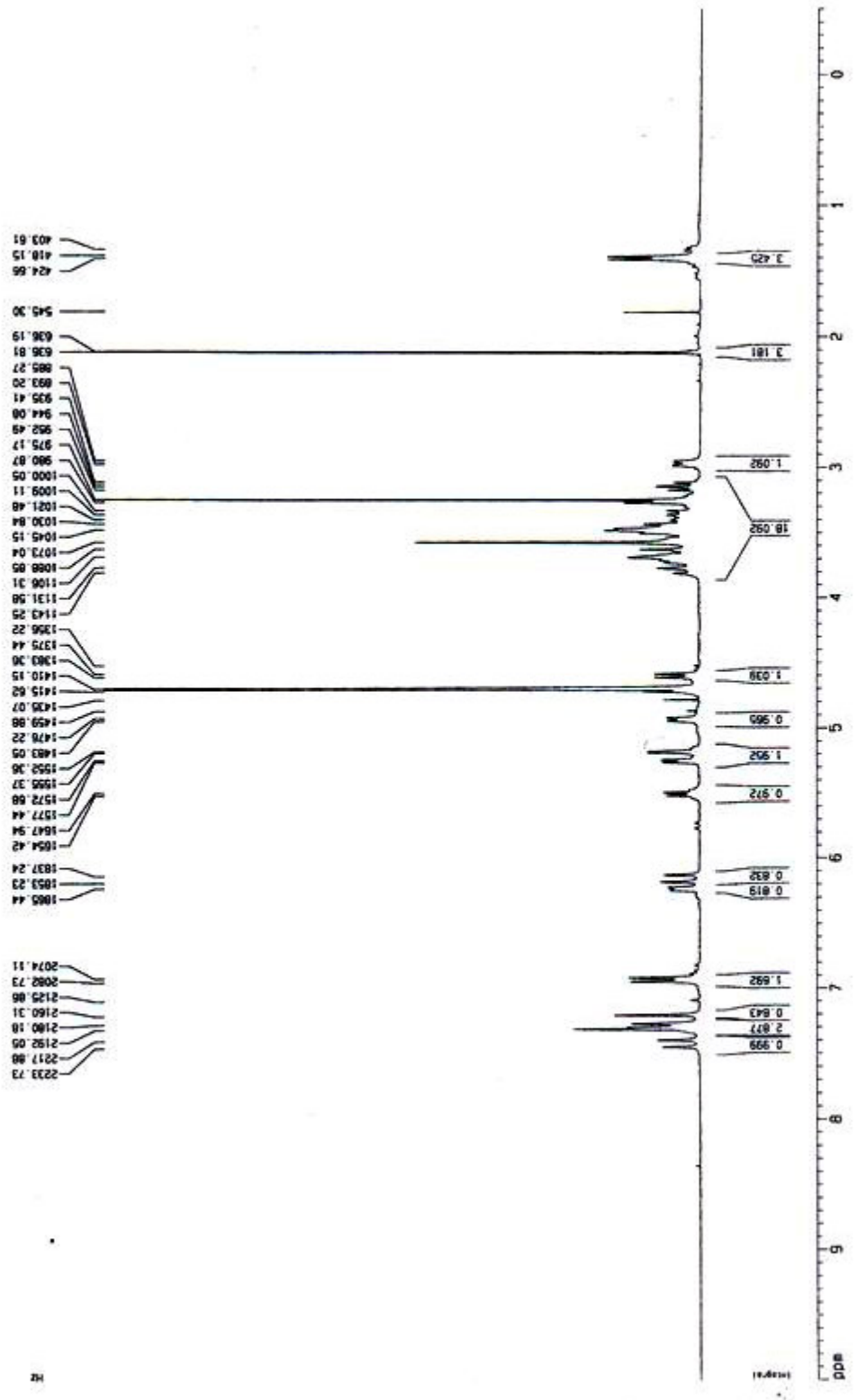


Figure 1 The 400 MHz ^{1}H NMR spectrum of *Plumeria* β-glucoside

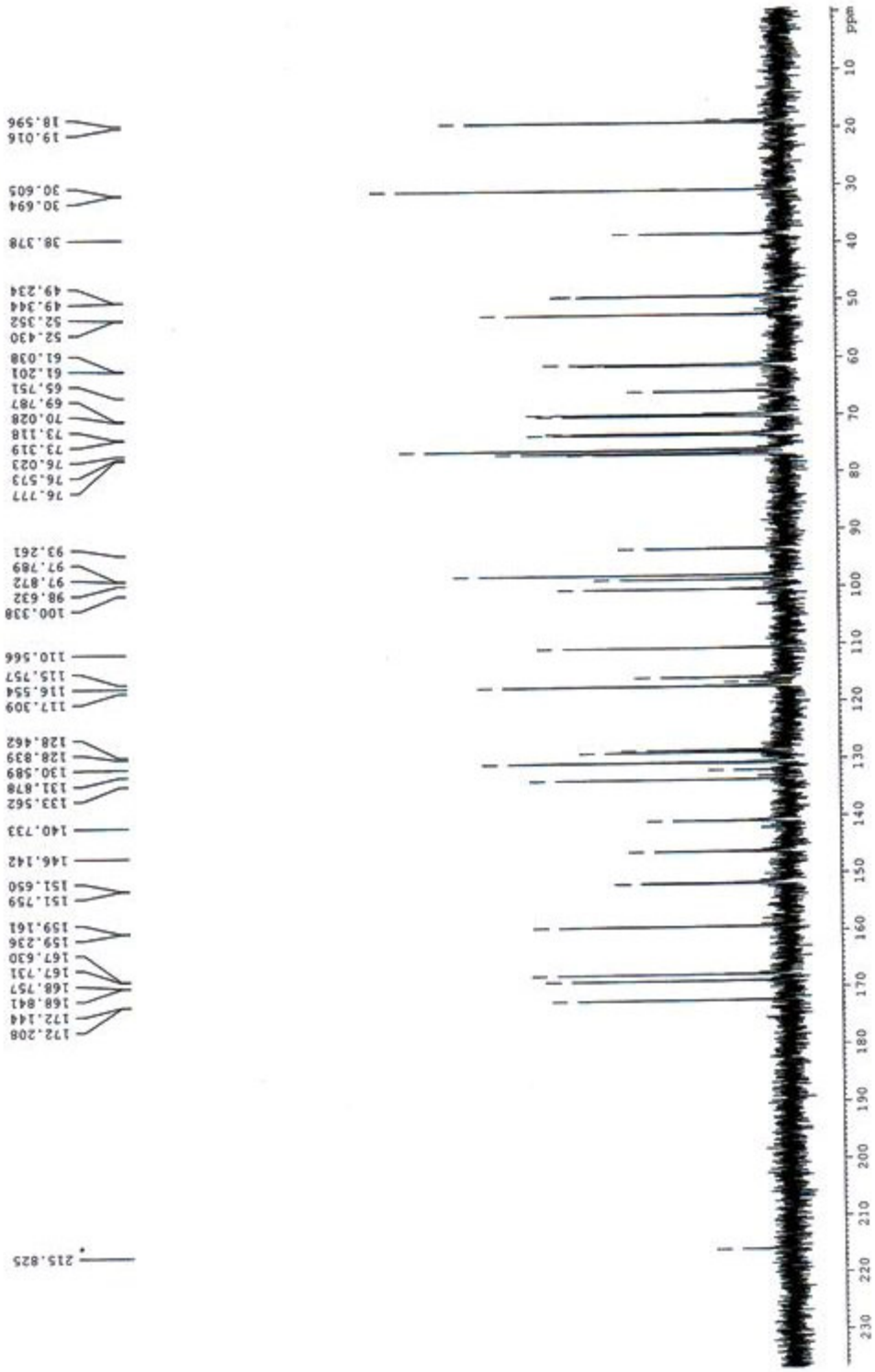


Figure 2 The 100 MHz ^{13}C NMR spectrum of *Plumeria* β-glucoside

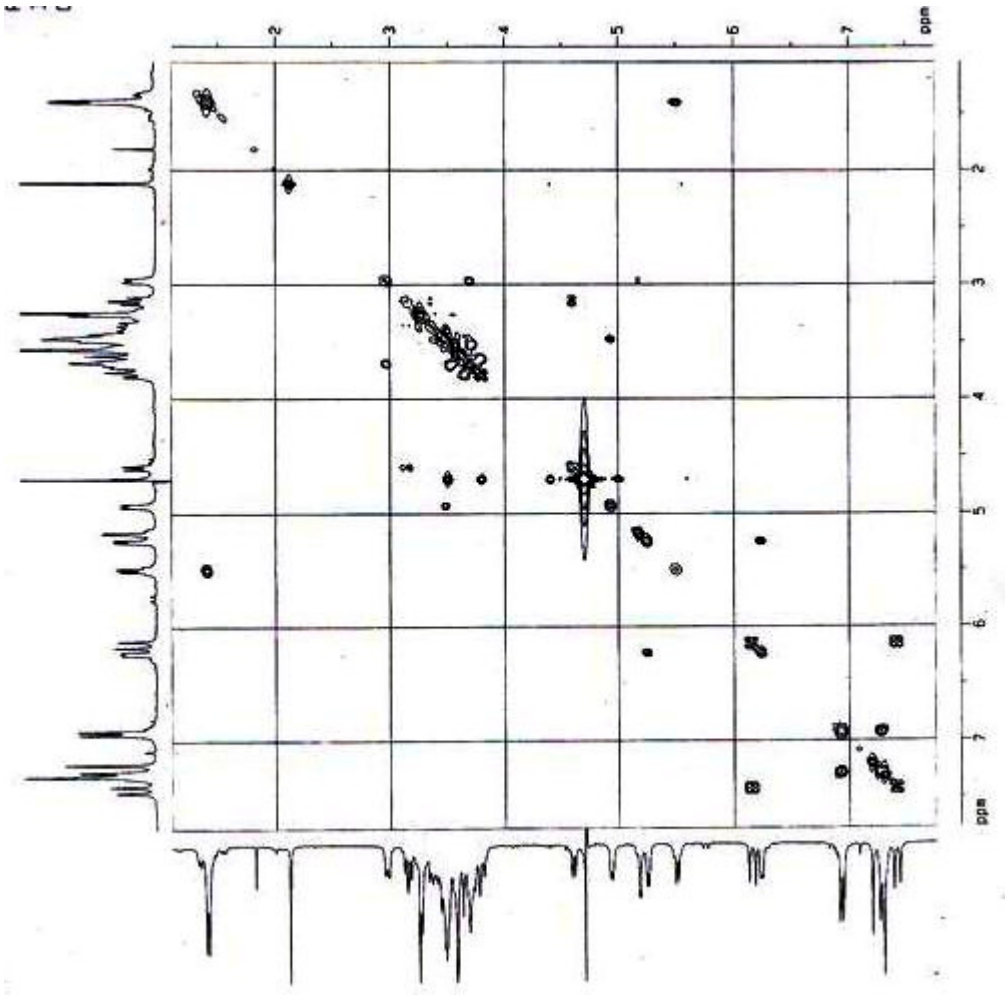


Figure 3 1H-1H COSY spectrum of *Plumeria* β-glucoside

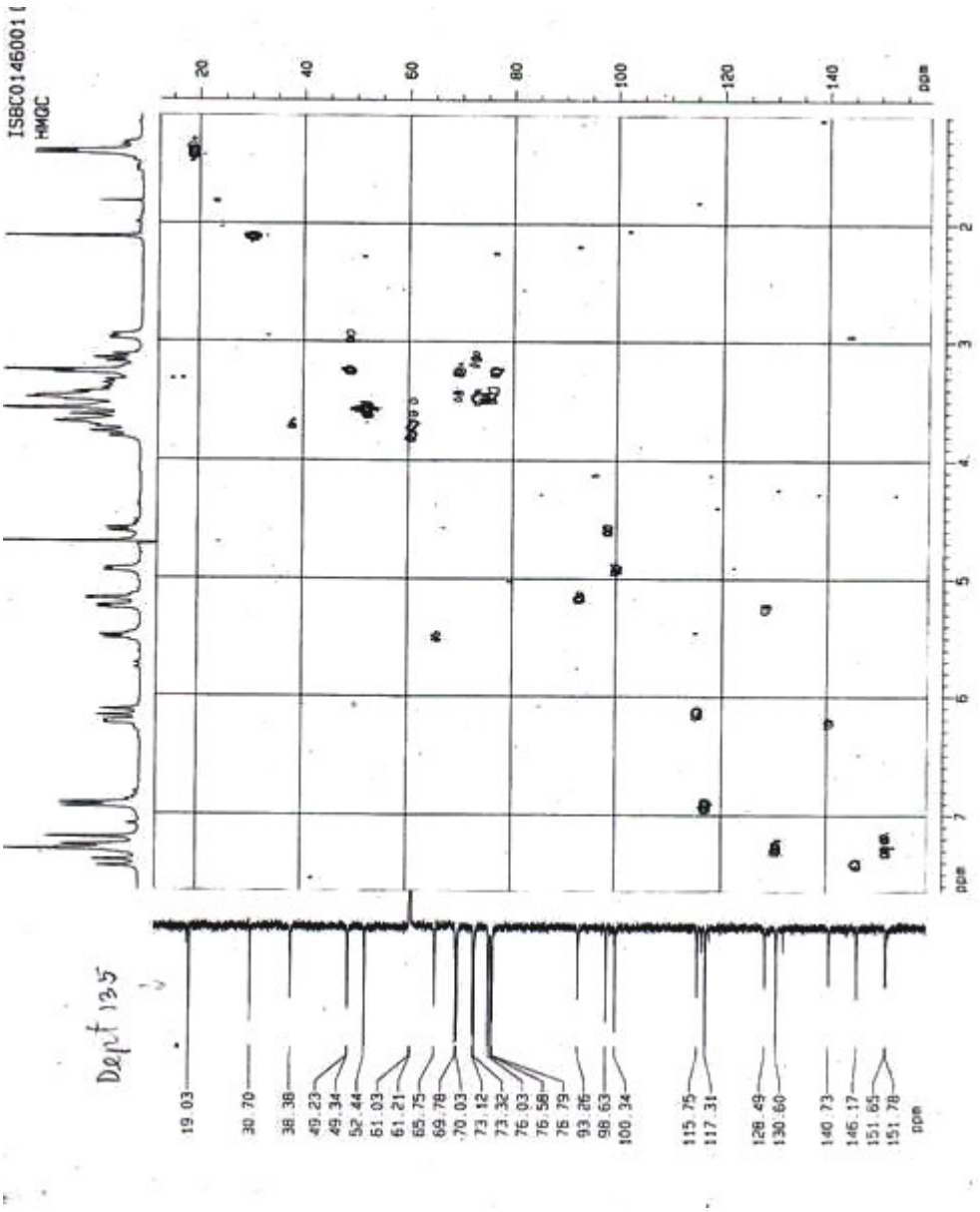


Figure 4 HMQC spectrum of *Plumeria* β-glucoside

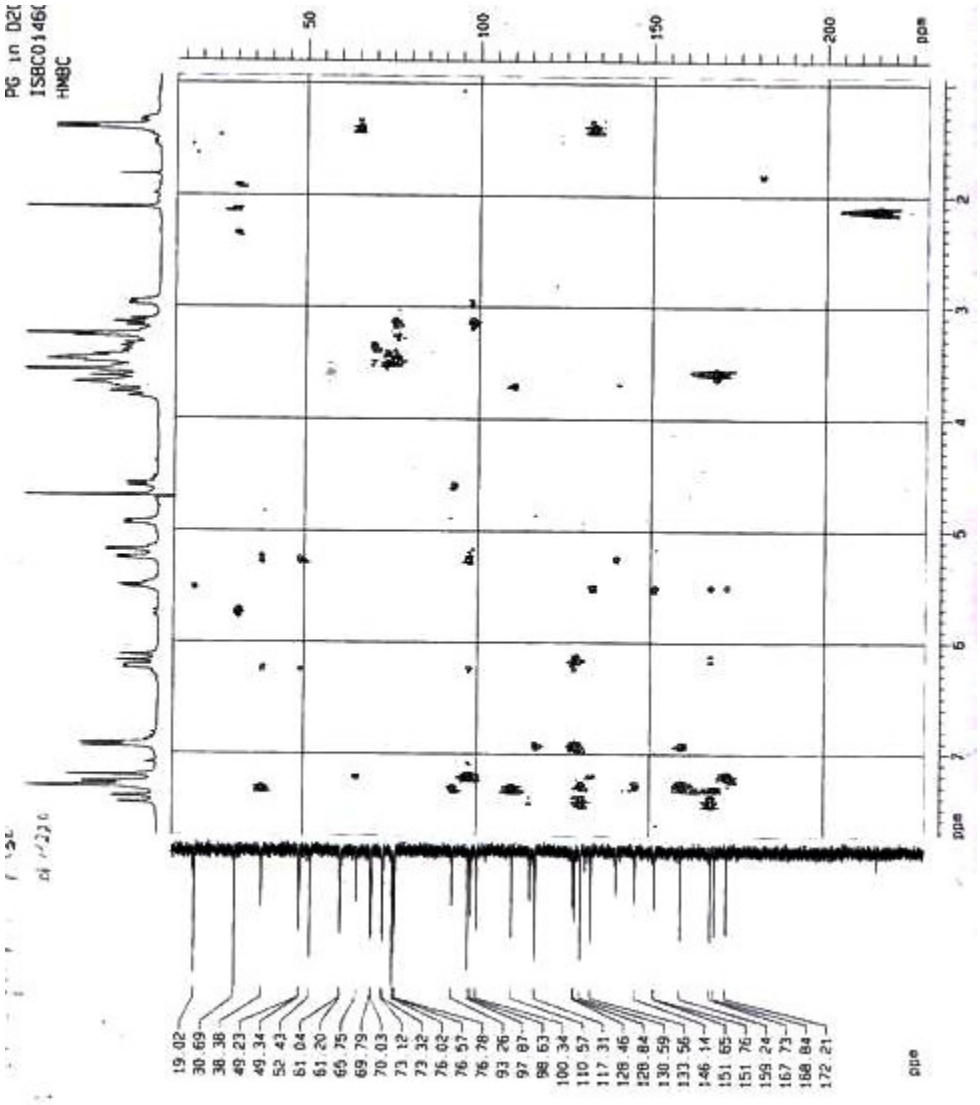
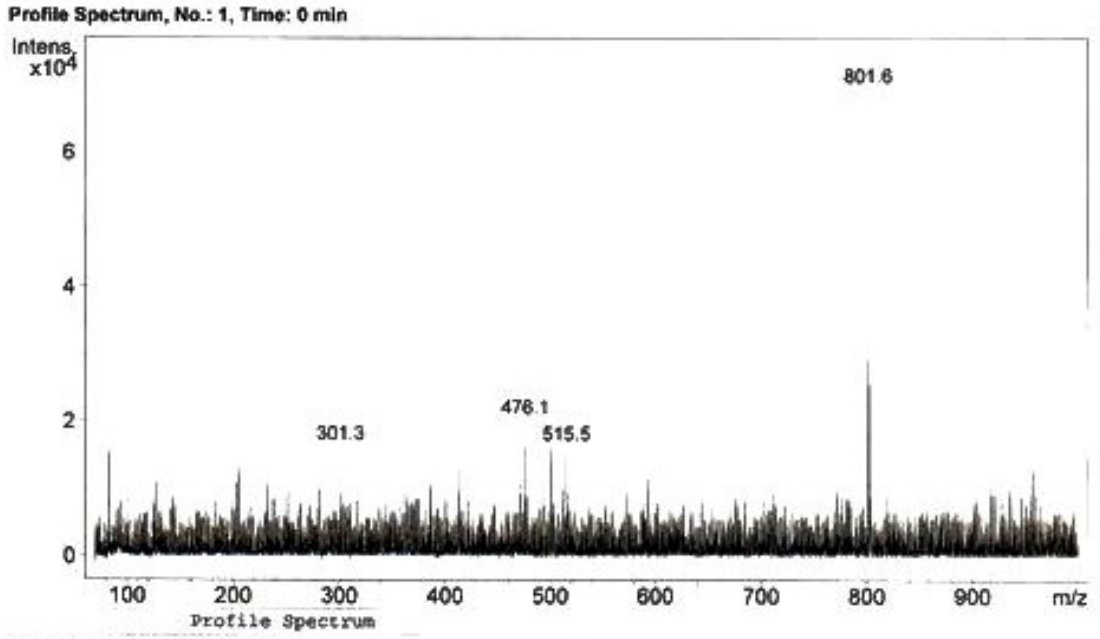


Figure 5 HMBC spectrum of *Plumeria* β-glucoside



MS Peak List (Profile Spectrum):

Mass	Intensity	Width	Mass	Intensity	Width	Mass	Intensity	Width
301.3	16370	0.25	501.5	15871	0.13	801.6	69476	0.25
476.1	20277	0.13	515.5	16304	0.31			

End of report

Figure 6 Accurate mass spectrum of *Plumeria* β-glucoside

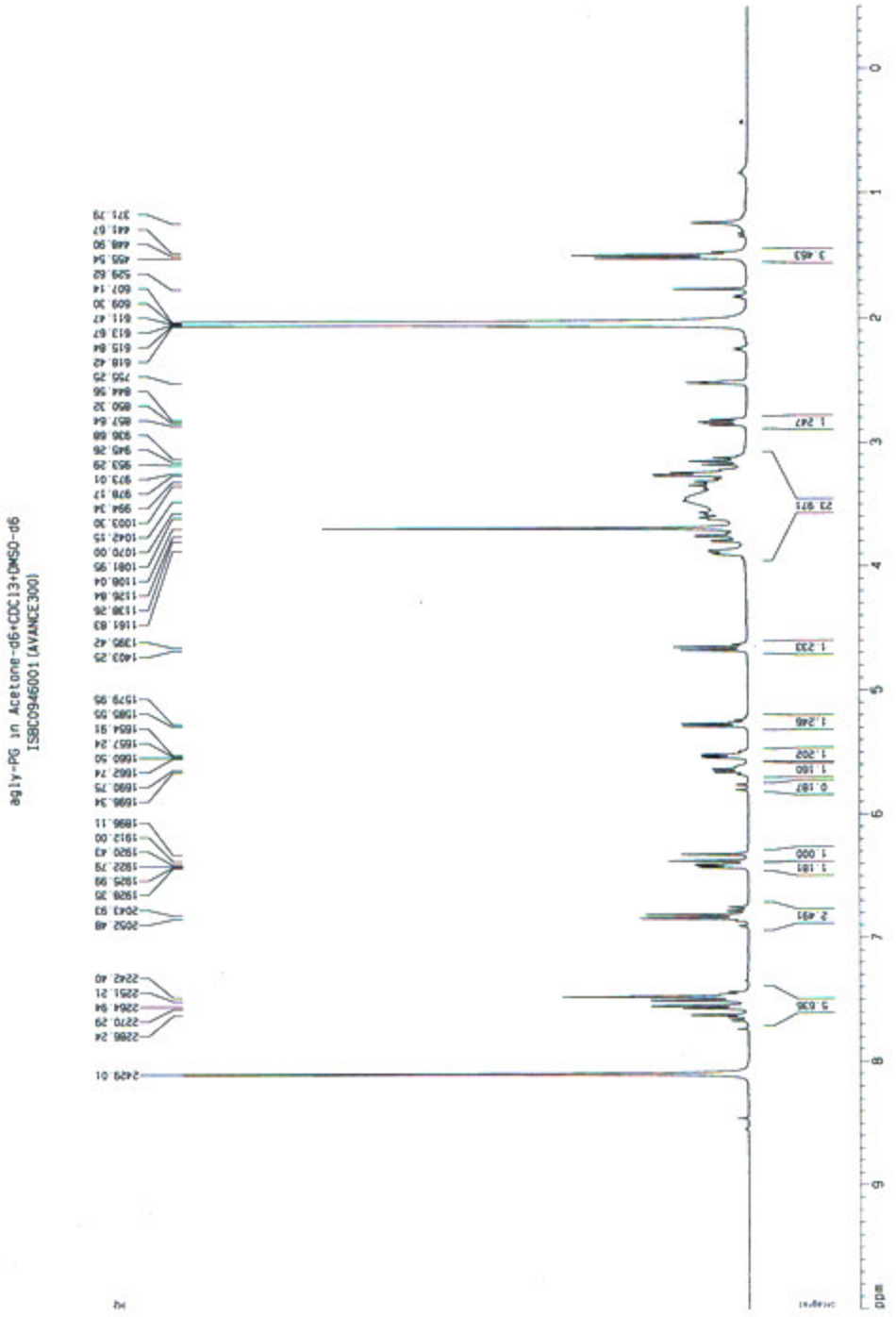


Figure 7 The 400 MHz ^{1}H NMR spectrum of aglycone of *Plumeria* β-glucoside

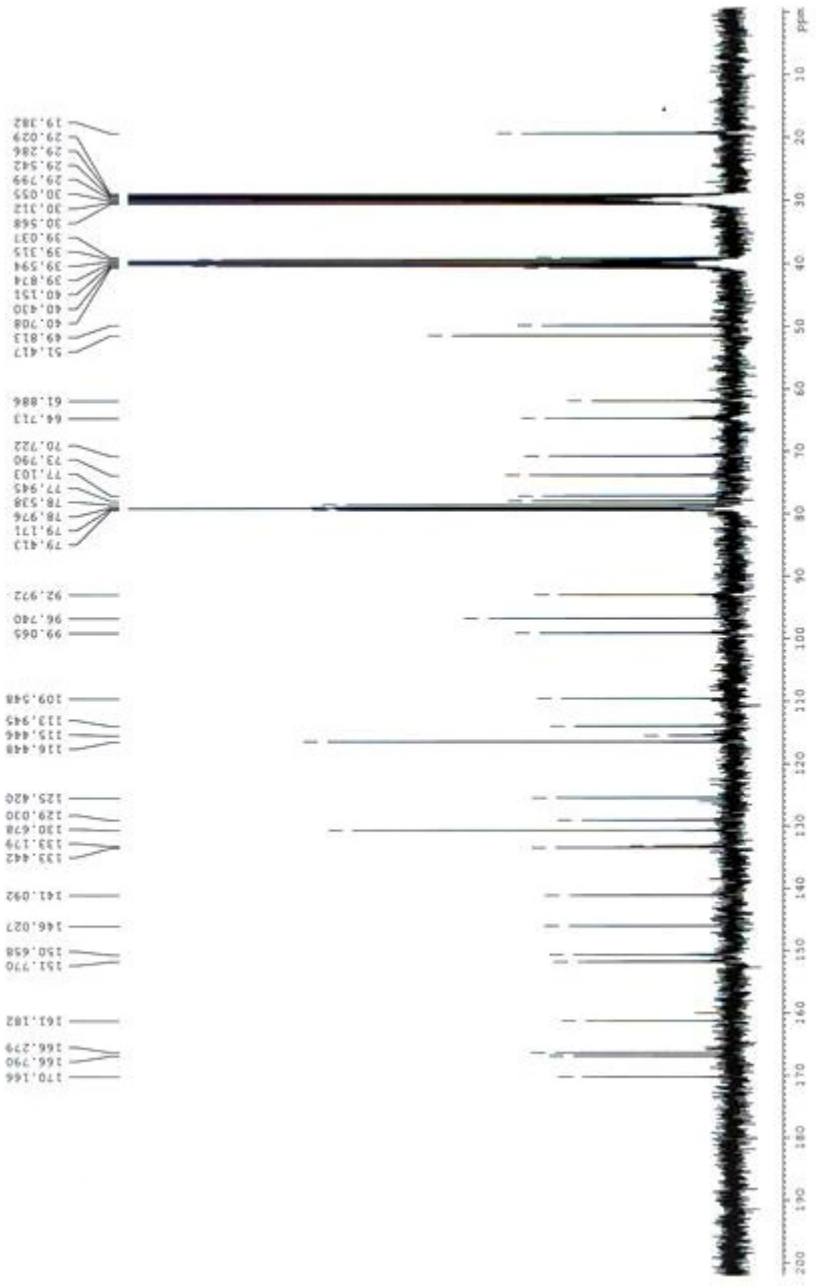


Figure 8 The 100 MHz ^{13}C NMR spectrum of aglycone of *Plumeria* β-glucoside

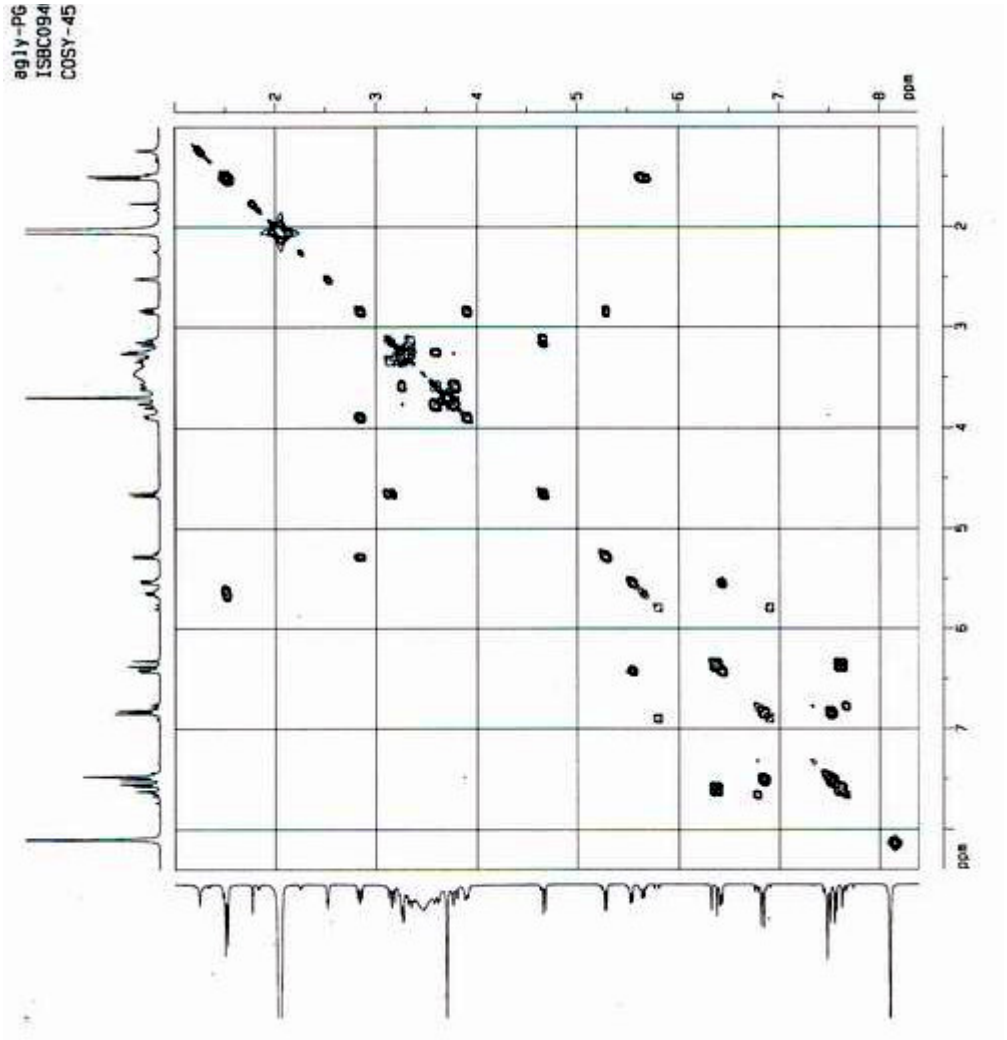


Figure 9 1H-1H COSY spectrum of aglycone of *Plumeria* β-glucoside

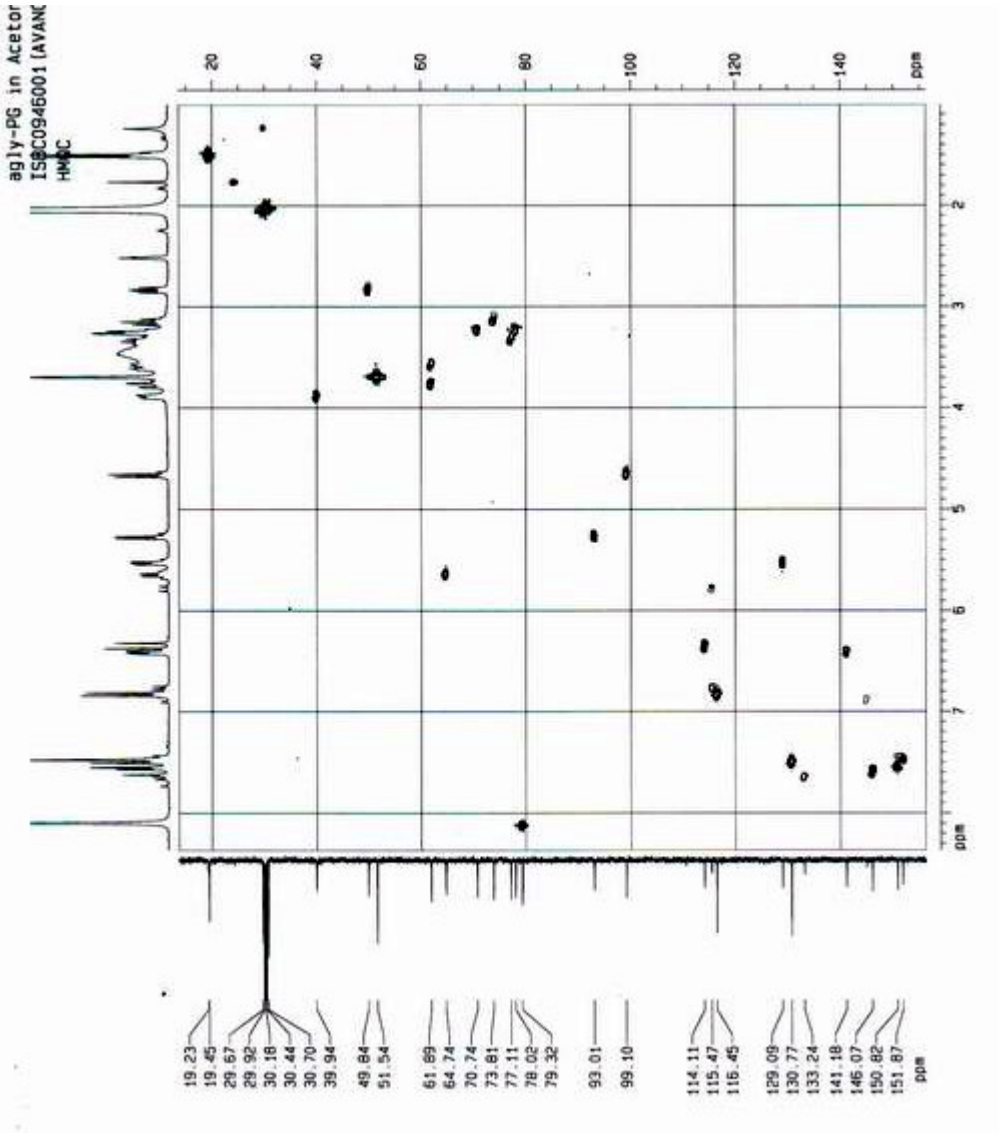


Figure 10 HMQC spectrum of aglycone of *Plumeria* β-glucoside

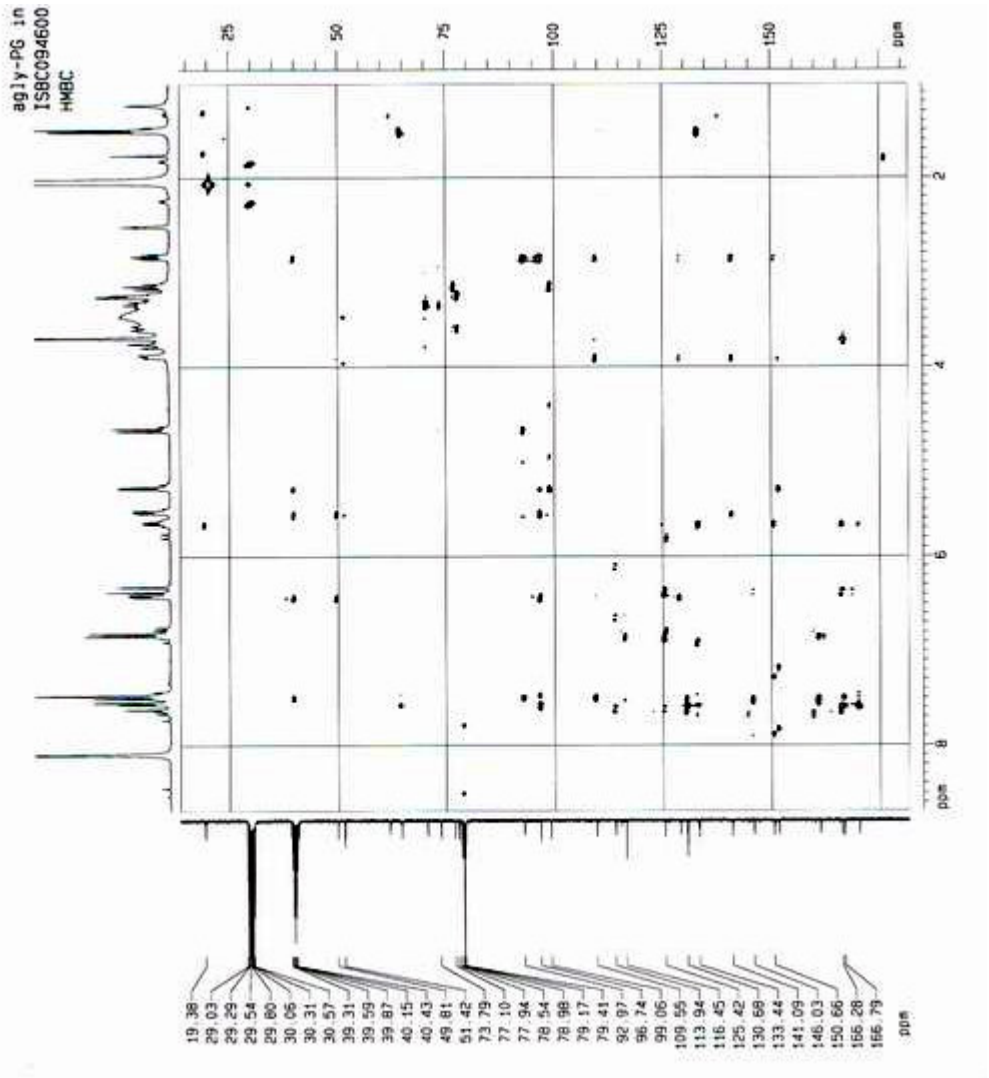


Figure 11 HMBC spectrum of aglycone of *Plumeria* β-glucoside

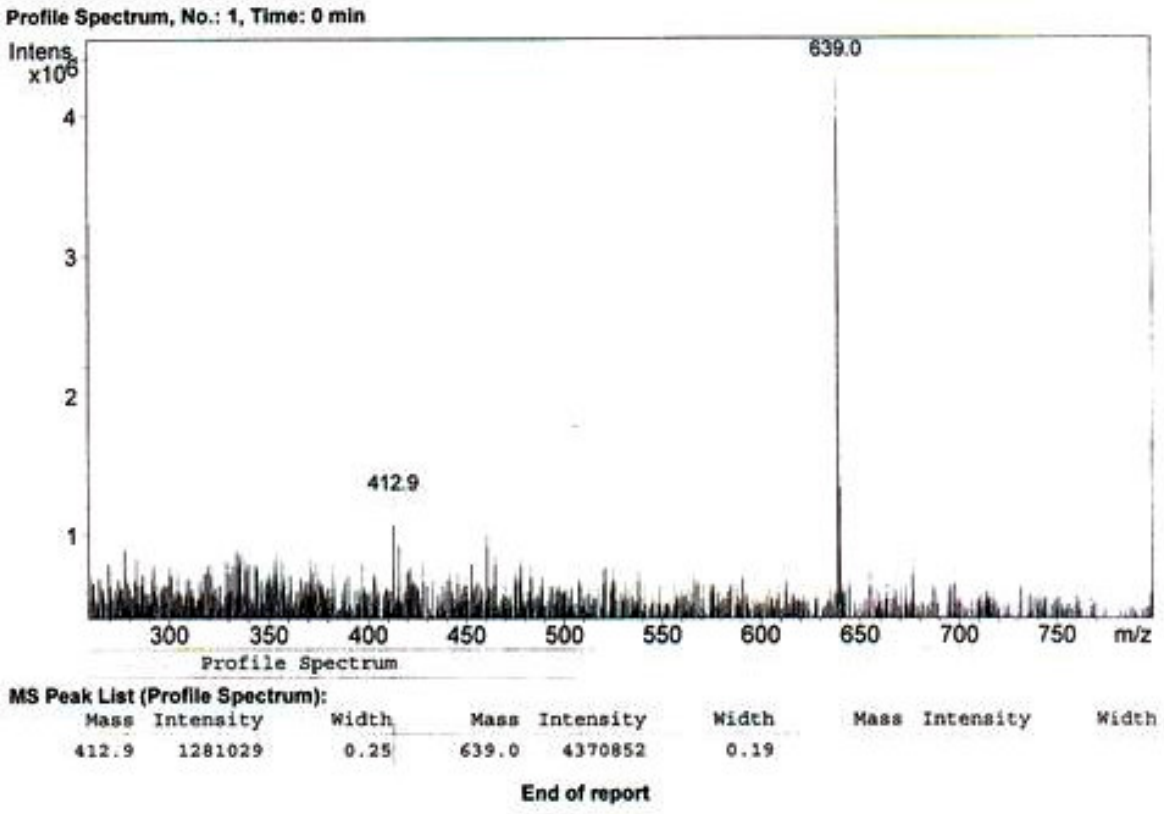


Figure 12 Accurate mass spectrum of aglycone of *Plumeria* β-glucoside

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