โปรตีนจากเมล็ดลูกเนียง *Archidendron jiringa* Nielsen. ที่มีฤทธิ์ยับยั้งแอลฟา-กลูโคซิเดส

นางสาว ปนัดดา วิรุฬห์บุญภัทร

PROTEIN FROM SEEDS OF DJENKOL BEAN *Archidendron jiringa* Nielsen. WITH ALPHA-GLUCOSIDASE INHIBITORY ACTIVITY

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ปนัดดา วิรุฬห์บุญภัทร: ฤทธิ์ยับยั้งแอลฟา-กลูโคซิเดสของโปรตีนจากเมล็ดลูกเนียง *Archidendron jiringa* Nielsen. ALPHA-GLUCOSIDASE INHIBITORY ACTIVITY OF PROTEIN FROM SEEDS OF DJENKOL BEAN *Archidendron jiringa* Nielsen. อ.ที่ปรึกษาวิทยานิพนธ์หลัก: รศ.ดร. พลกฤษณ์ แสงวณิช, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: อ.ดร. อภิชาติ กาญจนทัต, 73 หน้า.

สารยับยั้งเอนไซม์แอลฟา-กลูโคซิเดสตามธรรมชาติ มีหน้าที่ในการย่อยคาร์โบไฮเดรต ให้เป็นน้ำตาลโมเลกุลเดี่ยว นิยมใช้ในการรักษาโรคเบาหวานชนิดที่ 2 หรือโรคเบาหวานแบบ ไม่พึ่งพาอินซูลิน (Type II : non-insulin dependent diabetes) พืชในกลุ่มลิกูม (legumes) ได้รับ การรายงานว่าเป็นแหล่งพลังงานหลักที่มีโปรตีนและโพลีเปปไทด์ปริมาณสูง พร้อมทั้งมีสาร ออกฤทธิ์ทางชีวภาพที่สำคัญ หนึ่งในนั้นคือ ฤทธิ์ในการยับยั้งเอนไซม์แอลฟา-กลูโคซิเดส ใน การศึกษานี้สนใจแยกโปรตีนที่มีฤทธิ์ยับยั้งแอลฟา-กลูโคซิเดส จากเมล็ดพืช 2 ชนิด คือ เมล็ด ลูกเนียง (*Archidendron jiringa* Nielsen.) และเมล็ดสะตอ (*Parkia speciosa* Hassk.) พบ สารออกฤทธิ์ยับยั้งแอลฟา-กลูโคซิเดส ที่สกัดได้จากลูกเนียง *A. jiringa* โดยใช้ Tris-HCl เป็น บัฟเฟอร์ จากนั้นตกตะกอนโปรตีนด้วยเกลือแอมโมเนียมซัลเฟส 90% และแยกโปรตีนให้ บริสุทธิ์ด้วยเทคนิคทางโครมาโทกราฟี แบบสัมพรรคภาพ โดยใช้คอนเอเซฟาโรส (ConA Sepharose) เป็นวัฏภาคอยู่กับที่ พบสารที่แยกได้จากคอนเอเซฟาโรส มีกิจกรรมการยับยั้ง แอลฟา-กลูโคซิเดส โดยใช้ *p*-nitrophenyl α-D-glucopyranoside (PNPG) เป็น Substrate โดยให้ค่ากิจกรรมการยับยั้งที่ IC_{50} เท่ากับ 0.031 มิลลิกรัมโปรตีน จากการทดสอบหาความ บริสุทธิ์ของโปรตีนที่แยกได้ โดย SDS-PAGE พบสารยับยั้งแอลฟา-กลูโคซิเดสบริสุทธิ์ที่สกัด ได้ มีน้ำหนักโมเลกุล 35.7 กิโลดาลตัน โปรตีนที่แยกได้มีความเสถียรภาพต่ออุณหภูมิที่ 80°C เป็นเวลา 70 นาที ภาวะความเป็นกรด-ด่างที่เหมาะสมในการทำงานของตัวยับยั้งคือที่ pH 8.0- 10.0 และออกฤทธิ์ยับยั้งได้ดีเมื่อบ่มกับโลหะไอออน เช่น Cu^{2+}, Zn^{2+} และ Fe^{2+} สารออกฤทธิ์ ยับยั้งแอลฟา-กลูโคซิเดสที่สกัดได้ มีลำดับกรดอะมิโนใกล้เคียงกับเลคตินจากพืช *Dioclea guainensis* นอกจากนี้สารยับยั้งแอลฟา-กลูโคซิเดสที่แยกได้มีความจำเพาะในการจับกับ เอนไซม์แอลฟา-กลูโคซิเดส เมื่อยืนยันด้วยเทคนิค Surface plasmon resonance (SPR)

สาขาวิชา   เทคโนโลยีชีวภาพ   ลายมือชื่อนิสิต...
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PANADDA VIROUNBOUNYAPAT: ALPHA-GLUCOSIDASE INHIBITORY ACTIVITY OF PROTEIN FROM SEEDS OF DJENKOL BEAN *Archidendron jiringa* Nielsen. ADVISOR: ASSOC. PROF. POLKIT SANGVANICH, Ph.D., CO-ADVISOR: APHICHART KARNCHANATAT, Ph.D., 73 pp.

The inhibitors of α–glucosidase enzyme from natural resource which is responding to digest carbohydrates to monosaccharides. It has been used for the treatment of diabetes mellitus type II or non insulin dependent diabetes mellitus. Legumes were reported as a major source of proteins and polypeptides with important biological activities. One of the biological activities in legume is α–glucosidase inhibitory activity. This study was attempted to isolate α–glucosidase inhibitors from two leguminous plants: *Archidendron jiringa* Nielsen. (Djenkol bean) and *Parkia speciosa* Hassk. (Stink bean). The potential α–glucosidase inhibitor was found in the protein extraction from seeds of *A. jiringa* when used Tris-HCl buffer for extraction then precipitated with 90% ammonium sulfate, and purify with affinity chromatography using ConA Sepharose as stationary phase. The bound protein gave inhibitory activity against α–glucosidase using *p*-nitrophenyl α-D-glucopyranoside (PNPG) with the IC_{50} value of 0.031 mg protein. The molecular mass of the purified protein was 35.7 kDa, as estimated by SDS-PAGE. The protein was thermostable up to 80 ^{o}C for 70 min and showed an optimum activity within the pH range of 8.0-10.0. It has a high activity with divalent cations such as Cu^{2+}, Zn^{2+} and Fe^{2+}. The internal amino acid sequence of the protein showed that is similar to the sequences of lectin from *Dioclea guainensis*. Moreover, this α–glucosidase inhibitor has specific interaction with α–glucosidase enzyme which was confirmed by Surface Plasmon Resonance (SPR) technique.

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                                         Co-advisor's Signature _____

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CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

%	percentage
°C	degree celsius
μg	microgram
μl	microlitre
A	Absorbance
BLAST	Basic local alignment search tool
BSA	bovine serum albumin
ConA Sepharose	Concanavalin A Sepharose
cm	centimeter
Da	Dalton
EDTA	Ethylenediamine tetraacetic acid
ESI/MS/MS	Electrospray ionisation/Mass spectrometry/Mass spectrometry;
g	gram
hr	hour
IC_{50}	The half maximal inhibitory
kDa	kilodaton
l	litre
LC/MS/MS	Liquid Chromatography/Mass Spectrometry/Mass Spectrometry
M	molar
mA	milliampere
mg	milligram
min	minute
ml	milliliter
mM	millimolar
MW	molecular weight
N	normal
nm	nanometer
NaCl	Sodium chloride
PAGE	polyacrylamide gel electrophoresis

PNP	*p*-nitrophenol
PNPG	*p*-nitrophenyl-α-D-glucopyranoside
rpm	revolution per minute
SDS	sodium dodecyl sulfate
TBS	0.15 M NaCl / 20 mM Tris-HCl buffer, pH 7.2
TEMED	*N,N,N',N'*-tetramethyl ethylenediamine
TFA	Trifluoroacetic acid
Tris	Tris(hydroxymethyl)aminomethane
U	Unit activity
V	Volt
V/V	volume by volume
W/V	weight by volume

CHAPTER 1

INTRODUCTION

Diabetes mellitus (types 1 and 2) is recognized as a serious global health problem, often resulting in substantial morbidity and mortality. Type 2 diabetes mellitus is a group of disorders characterized by hyperglycemia and associated with microvascular (i.e., retinal, renal, possibly neuropathic), macrovascular (i.e., coronary, peripheral vascular), and neuropathic (i.e., autonomic, peripheral) complications. Unlike type 1 diabetes mellitus, the patients are not absolutely dependent upon insulin for life, even though many of these patients ultimately are treated with insulin. The management of type 2 diabetes mellitus often demands combined regimens, including diet and/or medicines, including sulfonylurea, and biguanide, as well as insulin. Besides the use of multiple approaches, α-glucosidase inhibitors are one of the alternative therapeutic approaches. The inhibition of intestinal α-glucosidases, would delay the digestion and absorption of carbohydrates and consequently suppress postprandial hyperglycemia (Puls *et al*., 1977). Furthermore, other benefits of α-glucosidase inhibitors, such as reducing triglycerides (Lebowitz, 1998) and postprandial insulin (Johnston *et al*., 1994) levels and anti-HIV activity (Bridges *et al*., 1994; Fischer *et al*., 1995; Fischer *et al*., 1996; Fischer *et al*., 1996) have been reported.

Herbal medicine, also called botanical medicine or phytomedicine, refers to the use of any plant's seeds, berries, roots, leaves, bark, or flowers for medicinal purposes. Long practiced outside of conventional medicine, herbal treatments are. It is becoming more interesting as up-to-date analysis and research show their value in the treatment and prevention of disease. Plants had been used for medicinal purposes long before recorded history. Recently, the World Health Organization (WHO) estimated that 80% of people worldwide rely on herbs. Increasing public dissatisfaction with the cost of prescription medications, combined with an interest in returning to natural or organic remedies, has led to an increase in the use of herbal medicines (Bruneton, 1995).

α-Glucosidase is the amylase enzyme for digestion of polysaccharide and oligosaccharide to monosaccharides (Kakavanos *et al*., 2006), its contributing starch

and glycogen metabolisms in plant and animal tissue is characterized by the variety in substrate recognition. Inhibition of α-glucosidase decreases the blood glucose levels via delaying digestion of poly- and oligosaccharides to absorbable monosaccharides (McCulloch *et al*., 1983). Thus α-Glucosidase inhibitory testing is useful for screening plants which should be used for blood glucose treatment. The previous studies showed α-glucosidase inhibitory activity from cyanidin-3-galactoside, a natural anthocyanin was an α-glucosidase inhibitor and could be used in combination with acarbose for treatment of diabetes (Adisakwattana *et al*., 2009). Some substances have been developed to phamarceutical, such as acarbose (glucobay®) from α-glucosidase microbial bacteria *Actinoplane* sp.5 (Shinoda *et al*., 2006)*,* voglibose (basen®) from *Streptomyces hygroscopicus* var. *limoneus* (Chen *et al*., 2006) and miglitol (glyset®) from *S. roseochromogenus* (Lee *et al*., 2002).

Archidendron jiringa Nielsen (Fabaceae: Mimosoideae), the Jenkol bean or Luk Nieng tree, is a leguminous tree that is found in Indonesia, Malaysia and Thailand, and is economically important with diverse uses, including as a vegetable (young shoots) and pulse or food flavoring agent (seeds), medicine (leaves), source of dye for silk (pods) and timber for craft work and firewood (Ong and Norzalina, 1999). Given the abundance of this commercial species, and especially the abundant seed production (1000-4000 seeds per tree per year). From this point of view, many efforts have been made to search for more effective and safe inhibitors of α-glucosidase from natural materials to develop physiologically functional foods to treat diabetes mellitus. The aim of this research was to study the α-glucosidase inhibitor activity of *A. jiringa* seeds in relation to their proteinaceous content.

CHAPTER ll

LITERATURE REVIEWS

2.1 Diabetes Mellitus

2.1.1 Introduction

Diabetes, a health problem reaching epidemic proportions, is a disease where the body either produces little or no insulin, or where the body becomes progressively resistant to the action of insulin. This disease affects more than 200 million people worldwide (Zimmet and McCarty 1995), including more than 16 million people in the United States (Harris *et al*., 1998). There are two types of Diabetes - Type 1, formerly designated as Juvenile Diabetes; and Type 2, formerly designated as Adult Onset Diabetes. Type 1 diabetes accounts for 10% of all diabetes patients and results from a decreased ability to produce insulin due to a poorly understood autoimmune disease of the pancreas. Type 2 diabetes, accounting for 90% of diabetes patients is a disease of insulin resistance and abnormal carbohydrate metabolism (Chakrabarti and Rajagopalan, 2002). Chronic complications include neuropathy, accelerated atherosclerosis, andretinopathy, nephropathy resulting in end-stage renal disease, premature cardiovascular mortality, blindness, and amputation (DeFronzo and Ferannini, 1991; Gerich, 1977). The incidence of the disease in the world's population is driven by type 2 diabetes, currently with 5% of the world population having diabetes. The global cost of treating diabetes and its complications could reach one trillion US dollars annually by the year 2025 (Chakrabarti and Rajagopalan, 2002). Type 2, diabetes mellitus (DM), has both environmental and genetic causes. The primary pathogenic factors contributing to type 2 diabetes mellitus are decreased insulin secretion and insulin resistance resulting from defects within the pancreatic beta-cells, skeletal muscle, and the liver (Clark, 1998). These two abnormalities vary between different type 2 diabetic patients. Impaired insulin secretion is primarily found in lean diabetic patients and is due to insufficiencies of the pancreatic beta-cells. Impaired insulin sensitivity is predominantly found in obese diabetics and occurs at the liver and skeletal muscle tissue level (DeFronzo, 1988). The pathophysiology of type 2 DM is as follows:

(a) The pancreatic beta cells are unable to respond to excess glucose with appropriate

insulin secretion.

(b) An increased demand for insulin due to induced insulin resistance factors (e.g., obesity) begins.

(c) A compensation by the beta cells to increase insulin secretion results in maintenance of normal glucose levels.

(d) A gradual increase in insulin resistance and a gradual decrease of insulin secretion results in suppression of hepatic glucose output and impaired glucose tolerance appear.

(e) As insulin resistance increases, there is a simultaneous increase in hepatic glucose synthesis leading to fasting hyperglycemia.

(f) Concurrently, the pancreas fails to compensate for the increased demand of insulin and further contributes to hyperglycemia (Gerich, 1996).

Most DM patients suffer from visceral obesity and have high circulating levels of cholesterol, triglycerides, and low levels of high density cholesterol, which are factors known to contribute to the development of cardiovascular disease (Donahue, 1994). Separate from the above listed complications, changes in the electrical and mechanical properties of the heart can contribute to diabetic cardiopathy (Kannel and McGee, 1979). The most devastating consequences of complications include lower-limb amputation, loss of vision, myocardial infarction, end stage renal failure, and death (Chakrabarti and Rajagopalan, 2002).

2.1.2 History

The first recorded description of diabetes mellitus dates back to the Ebers papyrus in Egypt around 1500 BC and it has also been noted in almost all of the ancient culture from Asia to Europe to the Americas (Soumyanath, 2005). The term diabetes mellitus describes a metabolic disorder of multiple aetiologies characterized by chronic hyperglycemia with disturbance of carbohydrates, fat and protein metabolism (Alberti *et al*, 1998). Diabetes is a chronic disease that occurs when the pancreas fails to produce sufficient insulin, or when the body cannot effectively use the insulin it produces. Since insulin is the main hormone that regulates blood glucose levels, hyperglycemia is a common result of uncontrolled diabetes, which over time can lead to resious damage to many of the body's systems especially the nerves and blood vessels (WHO, 2006).

According to the World Health Organization (WHO) (2006), there are more than 180 million people worldwide suffering from diabetes and this number is likely to double by 2030. In 2005, an estimated 1.1 milion people died from diabetes, with

approximately 80% of these deaths occurring in poor and developing countries. Almost half of these deaths were in people under the age of 70 years with 55% being women. The WHO estimates that diabetes related deaths will increase by more than 50% in the next 10 years if the disease is not given urgent attention. Most notably, diabetes deaths are projected to increase by over 80% in upper-middle income countries between 2006 and 2015. Diabetes and its complications therefore pose significant economic and public health consequences for individuals, families, health systems and countries (WHO, 2006).

2.1.3 Classification of diabetes mellitus

The first widely accepted classification of diabetes mellitus was published by WHO in 1980 and modified in 1985 (Alberti *et al*, 1998). An expert committee proposed two major classes of diabetes mellitus and named them insulin dependent diabetes mellitus (IDDM) or type I and non-insulin dependent diabetes mellitus (NIDDM) or type II. A new class of malnutrition-related diabetes mellitus (MRDM) was introduced including other types like impaired glucose tolerance and gestational diabetes mellitus (GDM) (Alberti *et al*., 1998) (Table 1). The proposed classification encompasses both clinical stages and aetiological types of diabetes mellitus and other categories of hyperglycaemia. The clinical stages reflect that diabetes, regardless of its aetiology, progresses through several clinical stages during its natural history. People who have or who are developing diabetes mellitus can be categorized by a stage according to the clinical characteristics, even in the absence of information concerning the aetiology (Alberti *et al*, 1998). Diabetes mellitus is best classified into four major disease symdromes.

2.1.3.1 Type I diabetes (previously know as insulin-dependent or childhood-onset) is characterized by a lack of insulin production. It indicates the processes of beta-cell (β-cell) destruction that may ultimately lead to full diabetes where exogenous insulin is required to prevent the development of ketoacidosis, coma and death. It is usually characterized by the presence of anti-glutamic acid decarboxylase (anti-GAD), islet cell or insulin antibodies which point to the auithtoimmune processes that led to beta-cell destruction (Alberti *et al*., 1998). Without daily administration of insulin, Type I diabetes is rapidly fatal. Symptoms include excessive urine production (polyuria), thirst (polydipsia), constant hunger despite patients having a voracious appetite (polyphagia), weight loss, vision changes and fatigue (WHO, 2006). There are no

clear symptoms of diabetes and many people may not even know they are suffering from the disease until it is too late.

2.1.3.2 Type II diabetes (formerly called non-insulin-dependent or maturity/ adult-onset) results from the body's inability to use insulin which results in its accumulation in the fat cells (WHO, 2006). It is the most common form of diabetes and is characterized by disorder of insulin action and insulin secretion and either one can be the predominant feature (Alberti *et al*, 1998). Type II diabetes comprises 90% of the cases of diabetes in people around the world, and it is largely the result of excessive body weight and physical inactivity. Symptoms may be similar to those of Type I diabetes, but are often less marked. As a result, the disease tends to go undiagnosed for several years, until severe complications have already arisen. Until recently, this type of diabetes was seen only in adults but it is now worryingly also occurring in obese children due to the lack of physical activity and an increase intake of high sugar diets (WHO, 2006).

Type II Diabetes is characterized by fasting and postprandial hyperglycemia and relative insulin insufficiency. I left untreated hyperglycemia may cause long-term microvascular and macrovascular complications, such as nephropathy, neuropathy, retinopathy and atherosclerosis (Jain and Saraf, 2008). In the early stages of the disease, insulin resistance in the peripheral tissues like the muscle and fat is associated with a compensatory increase in insulin production by pancreatic β-cells. This increase in secretion of insulin initially promotes glucose utilization in the peripheral tissue and decrease hepatic gluconeogenesis. Unfortunately fasting insulin levels progressively increase in a step-wise manner until the β-cells can no longer compensate for toe increased insulin resistance and subsequently fail viz. It is the combination of β-cell failure and associated loss of insulin that eventually promotes the severe hyperglycemia evident inthese patients (Jain and Saraf, 2008).

Treatment of a type-II diabetes patient may be simple i.e. increased physical activity to reduce weight (and consequently insulin resistance), reduce intake of dietary fat and adequate intake of complex carbohydrates and fibre which improves insulin action and secretion or more complex through medical intervention i.e. several commercially pharmaceuticals that either enhances insulin action or secretion. Of the two, the first treatment option is the most beneficial as drug therapy may be

associated with the term underlying and undersired effects like weight gain or hyperglycemia (Jain and Sarf, 2008).

Type I and type II diabetes were clearly thought to represent genetically independent disease but various lines of evidence suggest that there is a stronger genetic component in the etiology of type II diabetes (Creutzfeldt & Lefebvre, 1988). It is known that environmental factors play a part in the manifestation of the disease as well (van Tilburg et al., 2008).

Table 2.1 Classification of type I and type II diabetes

Mode of classification	Type I diabetes	Type II diabetes
Age at onset	Juvenlsie-onset diabete (JOD) Occurs predominantly in children and young adults	Maturity-onset diabetes (MOD) occurs predominantly in middle-aged or old people
Insulin dependence	Insulin-dependent diabetes (IDDM) usually, with periods without insulin dependence are not frequently observed shortly after the onset of diabetes	Non-insulin dependent diabetes (NIDDM) give insulin treatment for better control often advisable especially in younger patients

2.1.3.3 Type III or Gestational diabetes; this type of diabetes first occurs during pregnancy. During pregnancy the need for insulin appears to increase and gestational diabetes occurs at the late stages of pregnancy. This type of diabetes may go away once the baby has been born but type II diabetes may develop later in life, in woman who has had gestational diabetes.

2.1.3.4 Secondary diabetes; diabetes may develop as a consequence of the other disease or medication. Davidson (1991) listed some causes of secondary diabetes a term coined by Davidson (1991) as "other types" of diabetes, these pancreatic diseases (pancreatitis, surgery, cystic fibrosis), the use of other drugs not prescribed for the condition (contraceptive pills, diuretics, steroids), genetic syndromes (extremely rare but many have been described) and endocrine disease (Cushing's, acromegaly) (Davidson, 1991).

2.1.3.5 Other Forms

The other types of diabetes (some are not true diabetes, but may progress into diabetes with time) arise as a result of complications and are very uncommon forms of diabetes mellitus, but their underlying defect and disease process can be identified in a relatively specific manner and include genetic defect of the beta-cell, disease of the exocrine pancreas, endocrinopathies, drug or chemically induced diabetes and uncommon forms of immune-mediated diabetes (WHO, 2002).

2.1.4 Factors influencing the prevalence of diabetes

Many risk factors have been identified which influence the prevalence or incidence of diabetes type II (van Tiburg *et al*., 2008). Factors of particular importance are:

2.1.4.1 Age; age is the single most important variable influencing the prevalence of diabetes. Epidemiological studies show that prevalence increase with age above 40. Oddly there has also been recorded evident of declining incidence with old age in some countries in Europe and the Americas (Jain and Saraf, 2008).

2.1.4.2 Gender; while diabetes was believed to be more common in females than males, recent trends has shown an equal prevalence for both males and females. However, there may be an increase in the prevalence of the disease in men in the last decade (Jain and Saraf, 2008).

2.1.4.3 Country and place of residence; while there appears to a country-associated distribution of the disease, this is most likely skewed due to the population age i.e. due to the fact that diabetes is an age related disorder; countries with elderly populations have more cases of diabetes compare to developing countries with younger populations. In some traditional communities in developing countries diabetes is rare (Jain & Saraf, 2008). Diabetes is considered as a disease of urbanization and several studies have found that the prevalence is significantly higher in urban populations than in rural communities within the same country (Jain and Saraf, 2008).

2.1.4.4 Ethnicity; many studies have shown the impact of ethnicity on the prevalence of diabetes e.g. studies have shown that Maxican American have a 1.9 times higher prevalence of diabetes than Native Americans. A locally based study showed that diabetes was 4 times higher in Indian men than white men and twice as high in India women than white women (Jain and Saraf, 2008). When the traditional lifestyle among blacks was followed in the past, diabetes was virtually absent. Since the 1960s

studies in South Africa indicated that the majority of the diabetes cases were all entirely hospital based. A study conducted in Cape Town showed a prevalence rate of 3% in 1969 of the black community, since then WHO has indicated an increase of up to 8% in 1985 (Bourne *et al*., 2002). Currently in South Africa the prevalence rate has risen to almost 30% of the population due to urbanization (Bourne *et al*., 2002).

2.1.4.5 Socio-economic status and lifestyle; socio-economic deprivation associated with poor diet (unhealthy diets-high calorific/ saturated fats) and other adverse lifestyle factors are linked to high rates for diabetes (Jain and Saraf, 2008).

2.1.4.6 Obesity; it is clear that obesity is a risk factor associated with diabetes type II diabetes (Jain and Saraf, 2008), as the prevalence of both obesity and diabetes have grown concurrently in many developing and developed countries.

2.1.5 Genetics of type II Diabetes mellitus

The occurrence of type II DM is also genetically linked. However, unlike other genetic disorders, type two diabetes, is a multi-factorial disease with many gene loci (genotype-genetic constitution of a cell, an organism or an individual), each with a small to moderate effect contributing to the overall disease. With any genetic disease, expression of these genes are dependent on environmental factors (Phenotype-overall characteristics of an organism e.g. morphology, biochemical or physiological properties) (van Tilburg *et al*., 2008).

Defects in gene involved in insulin secretion or insulin action, such as insulin receptor substrate 1 (IRS1), the glucagons receptor, the sulphonylurea receptor (SUR), the peroxisome proliferators activated receptor-γ (PPAR-γ) and the MAPKBIP1 have all been observed. The mutations in these genes seem to be limited to a small percentage of type II diabetes patients or to a specific population (van Tilburg *et al*., 2008).

Qu et al (2008) described and identified a haplotype (genetic constitution of an individual's chromosome) in the leptin receptor (LEPR) gene which is associated with type II diabetes among the northern Chinese population. The LEPR gene has been considered as one of the genes associated with type II diabetes in many population studies in the last decade. The LEPR is a member of the cytokine receptor family, which plays a critical role in the regulation of energy balance including glucose metabolism and body weight by activating transcription (STAT) protein STAT3, 5 and 6.

Most recently genome wide scans in four American populations have indicated suggestive linkage to type II diabetes or impaired glucose homeostasis on chromosome 5, 12 and X in Caucasians, on chromosome 3 in Maxicans Americans and chromosome 10 in African Americans. In the eastern and south eastern Chinese Han population two loci in a region on chromosome 9 showed suggestive evidence for linkage to type II diabetes (Jain and Saraf, 2008).

2.2 Physiology of Glucose metabolism

2.2.1 Glucose metabolism

Like any machine the body requires fuel to provide/ produce energy for it to function. The fuels the body needs come from the food we eat, which are made up of carbohydrates (sugars and starch), proteins and fats. These carbohydrates, proteins and fats are split apart by digestive enzyme into their basic building blocks glucose, amino acids and fatty acids respectively (Krall and Beaser, 1989). There is a series of chemical reactions in the metabolic pathway which produces energy from glucose and this pathway is called the Krebs cycle coupled to the electron transfer chain which is present in practically all cells (Krall and Beaser, 1989).

From the three main source of energy, carbohydrates appear to be the most important. Once carbohydrates are broken down into glucose, the glucose enters the blood stream and comes into contact with all cells including those in the pancreas (pancreatic β-cells). Here it causes the secretion of insulin in response to increased glucose levels. The insulin produced and subsequently excreted then stimulates the cells, through the attachment to insulin receptors on the cell surface, which allows glucose to enter the cells and to be used as a direct energy source or if in excess for the remaining energy is stored as glycogen in the liver or muscles (Krall and Beaser, 1989). (For more detail, please see 2.4.2 below)

There are three stages that must occur in order to generate energy from these foodstuffs

2.2.1.1 The first stage; involves the breakdown of large molecules into smaller units, i.e. proteins are hydrolyzed to their twenty kinds of constituent amino acids, polysaccharides are hydrolyzed to simple sugars and fats are hydrolyzed to glycerol and fatty acids.

2.2.1.2 The second stage; the small units from the first stage are degraded to a few simple units that play a pivotal role in metabolism. Here most of the sugars, amino

acids and fatty acids are converted into acetyl units of acetyl CoA and a small amount of adenosine triphosphate (ATP) is generated.

2.2.1.3 The third stage; the third stage consists of the citric acid cycle and oxidative phosphorylation, the final pathway in the oxidation of fuel molecules. Acetyl units are completely oxidized at this stage to CO_2 and four pairs of electrons are transferred to NAD+ and FAD for each acetyl group that is oxidized. ATP is then generated as electrons flow from the reduced forms of these carriers to O_2 in a process known as oxidative phosphorylation. The bulk of ATP is generated in this stage (Stryer, 1988).

2.2.2 Pathophysiology

Appropriate treatment of Type II diabetes is dependent on the knowledge of the pathophysiology of the disease, the mechanisms underlying hyperglycemia and the efficacy of various oral agents an insulin to improve fasting or postprandial hyperglycemia (Codario, 2005). There is no single genetic defect that has been elucidated to explain the aetiology of the disease and thus it is said that the disease may result from a combination of multigenic, heterogeneous, complex and related causes (see 2.3.4 above). A small percentage (<10%) of individuals with monogenic causes of type II diabetes inherit two mutant genes from both parents (Codario, 2005).

To understand the disease, one needs to first understand the normal physiology of insulin action, which is its effect on glucose. Insulin is a protein made up of a long chain of smaller building blocks called amino acids. The β-cells have the ability to take 86 amino acids and hook them together to form a long chain of amino acids called proinsulin (Krall & Beaser, 1988). The proinsulin is packaged as secretory granules and within these granules the proinsulin is cleaved resulting in the production of insulin and a C-peptide which are released into the blood stream through the stimulation of β-cells. The C-peptide has no known function but it is released by β-cells into circulation and it has been useful as a marker to determine how much insulin is released (Krall and Beaser, 1988).

Insulin is released in two stages, during the stage, which is very rapid and occurs within 10 minutes after the signal is received, insulin is released from the β-cells (Krall and Beaser, 1988). The second stage is more complicated, a signal is sent to the nucleus of the β-cells which stimulates the insulin gene which in turns on the transcription of mRNA which transports the signal to the pert of the cell that is required to stimulate the production of insulin (Krall and Beaser, 1988). In people

without diabetes it is not always possible to raise the levels of blood glucose no matter what they eat because the insulin reserve is plentiful and is secreted in exactly the correct amount, this is however not the case with people suffering from diabetes, the reverse is quite true (Krall and Beaser, 1988).

Glucose transport is rate limiting for overall disposal under most normal physiological conditions. Out of the five types of glucose transporters (GLUT) identified, GLUT4 is the only protein referred to as insulin-sensitive glucose transporter (Codario, 2005). Patients with type II diabetes usually have normal GLUT4 levels but impaired glucose transport and this may indicate that is a flaw that exists in the insulin-influenced translocation of GLUT4 to the cell surface. This defective signaling pathway between the receptor and the transport stimulation results in insulin resistance in type II diabetes patients (Codario, 2005).

Fasting glucose levels are dependent on hepatic glucose production, basal insulin levels, insulin sensitivity and the level and duration of the previous prandial glucose. After a meal, elevated glucose levels stimulate insulin release from β-cells. The secreted insulin binds to cell surface receptors and two extra cellular α subunits bind to the insulin and transmit a signal to the two identical β subunits via the cell membrane (Codario, 2005). After the binding process the β subunit is phosphorylated increasing tyrosine kinase activity enhancing the phosphorylation of the various endogenous protein substrates (Codario, 2005). This results in a cascading sequence of reactions responsible for the synthesis of proteins and intracellular enzymes, which suppress glucose output and glucose uptake in the peripheral tissue is enhanced. Type II diabetes patients have multiple intracellular deficiencies related to the phosphorylation e.g. impaired ability to phosphorylate and to stimulate the association of insulin receptor stimulator-1 (IRS-1) with the P85 subunit of the PI-3 kinase, impaired phosphorylation of PI-3 kinase and impaired induction of GLUT4 translocation by PI-3 kinase. The glucose molecules than bind to the GLUT4 protein which facilitate the transport of glucose into or out of the cell (Figure 2.1).
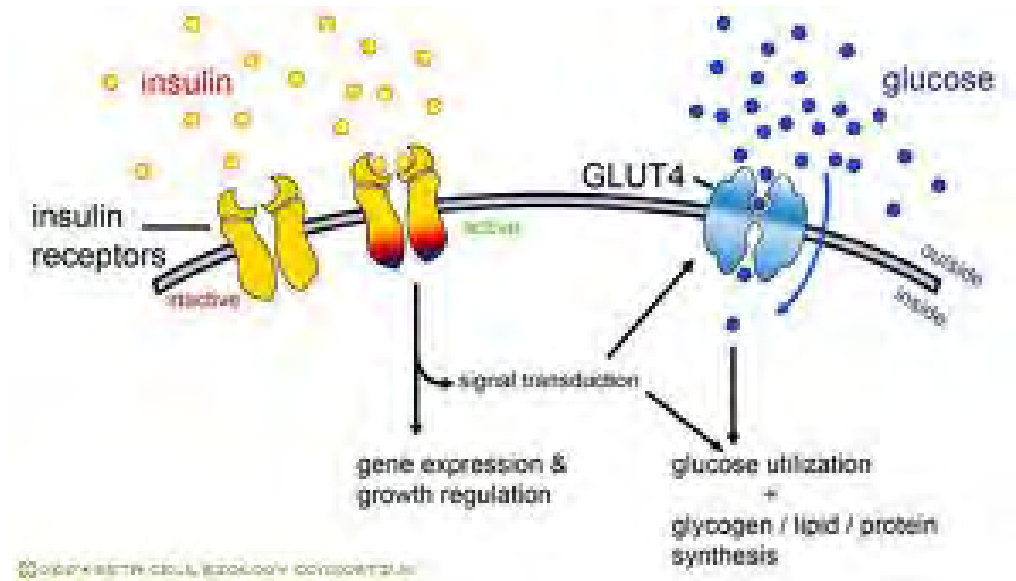
Figure 2.1 Insulin binding and activation of GLUT4. The binding of insulin to insulin receptor stimulate the glucose which leads to glucose utilization in the cell (http://www.dolly.biochem.arizona.edu).
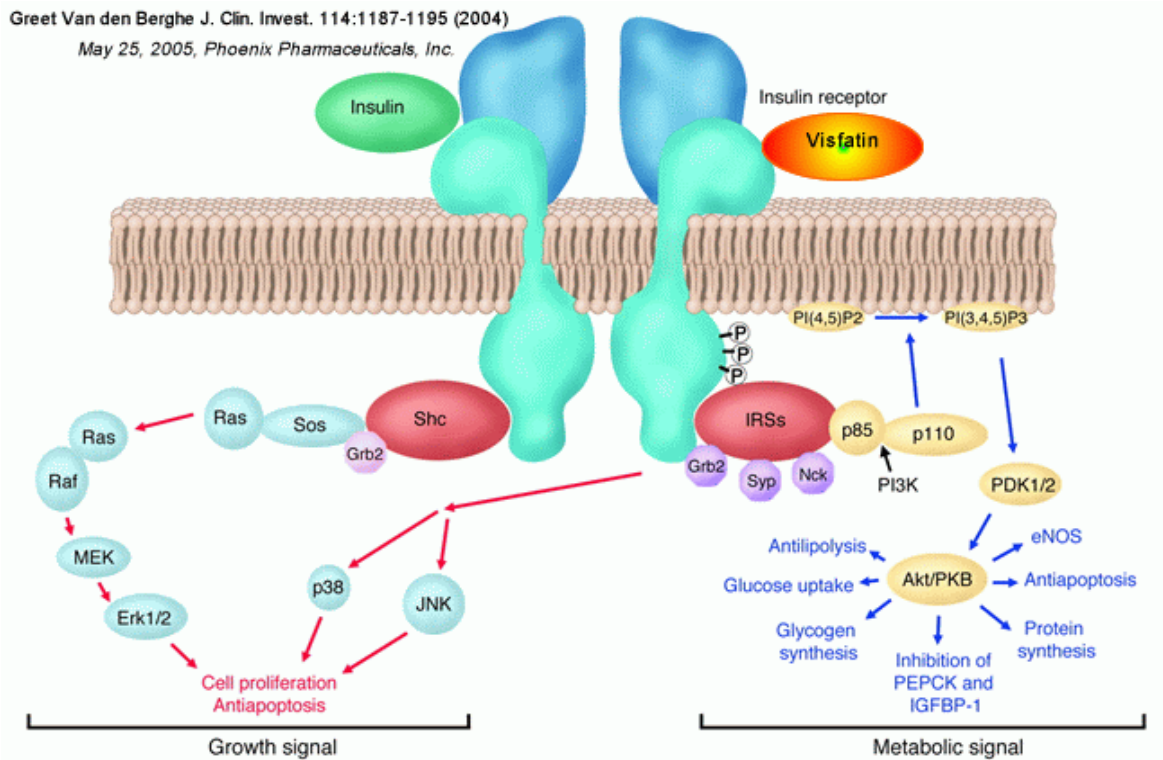
Figure 2.2 Phosphorylation and stimulation of IRS1 with P85 subunit. This diagram shows all the signal transduction and activation subunits that are responsible in the transduction of the insulin signal in the metabolic and growth pathway in cell.

2.3 Clinical recognition and diagnosis of diabetes mellitus

It is very difficult to physically diagnose diabetes mellitus because there are no external symptoms during the early stage and the clinician must feel very confident that the diagnosis is fully established since the consequences for the individual will be long term and life long. The requirements for proper diagnostic confirmation for a person presenting with severe symptoms and gross hyperglycaemia differ from those of an asymptomatic person with blood glucose levels just above that of the diagnostic cut-off level (7.0 mmol l^{-1} of fasting plasma glucose or 6.1 mmol l^{-1} for whole blood) (Alberti *et al*., 1998). The diabetes association of South Africa (2001) lists the following as signs that a person may be diabetic:

- Excessive appetite and thirst
- Increased amount and need of urine passed
- Weight loss (in the case of type I diabetes)
- Feeling of weakness and tiredness

- Itchiness of the skin

- Slow healing of cuts and wounds

- Frequent infections

- Hampered sight

- Pricking and dead feeling sensations in hands and feet

- Dizziness and occasional loss of balance

- Erectile dysfunction.

Diagnosing type II diabetes is often more problematic because the onset is very gradual and a person can have diabetes for many years before presenting with complication of the disease (McDowell *et al*., 2007). In order to diagnose type II diabetes one must first understand the sequence of that lead to the disease development (Diagram 2.1).

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┌─────────────────────────────┐
│   Genetic predisposition    │
└─────────────────────────────┘
                │
┌──────────────────────┐       │
│   Environmental and  │──────▶│
│   cultural factors   │       │
└──────────────────────┘       ▼
                ┌─────────────────────────────┐
                │      Insulin resistance     │
                └─────────────────────────────┘
                               │
                               ▼
                ┌─────────────────────────────┐
                │ Compensatory hyperinsulinaemia │
                └─────────────────────────────┘
                               │
                               ▼
                ┌─────────────────────────────┐
                │ Normal or impaired glucose tolerance │
                └─────────────────────────────┘
                               │
┌──────────────────────┐       │       ┌─────────────────┐
│ Genetic predisposition │     │       │  Glucotoxicity  │
└──────────────────────┘       │       └─────────────────┘
              ╲                ▼                ╱
                ┌─────────────────────────────┐
                │       Beta cell defect      │
                └─────────────────────────────┘
                               │
                               ▼
                ┌─────────────────────────────┐
                │       Type 2 diabetes       │
                └─────────────────────────────┘
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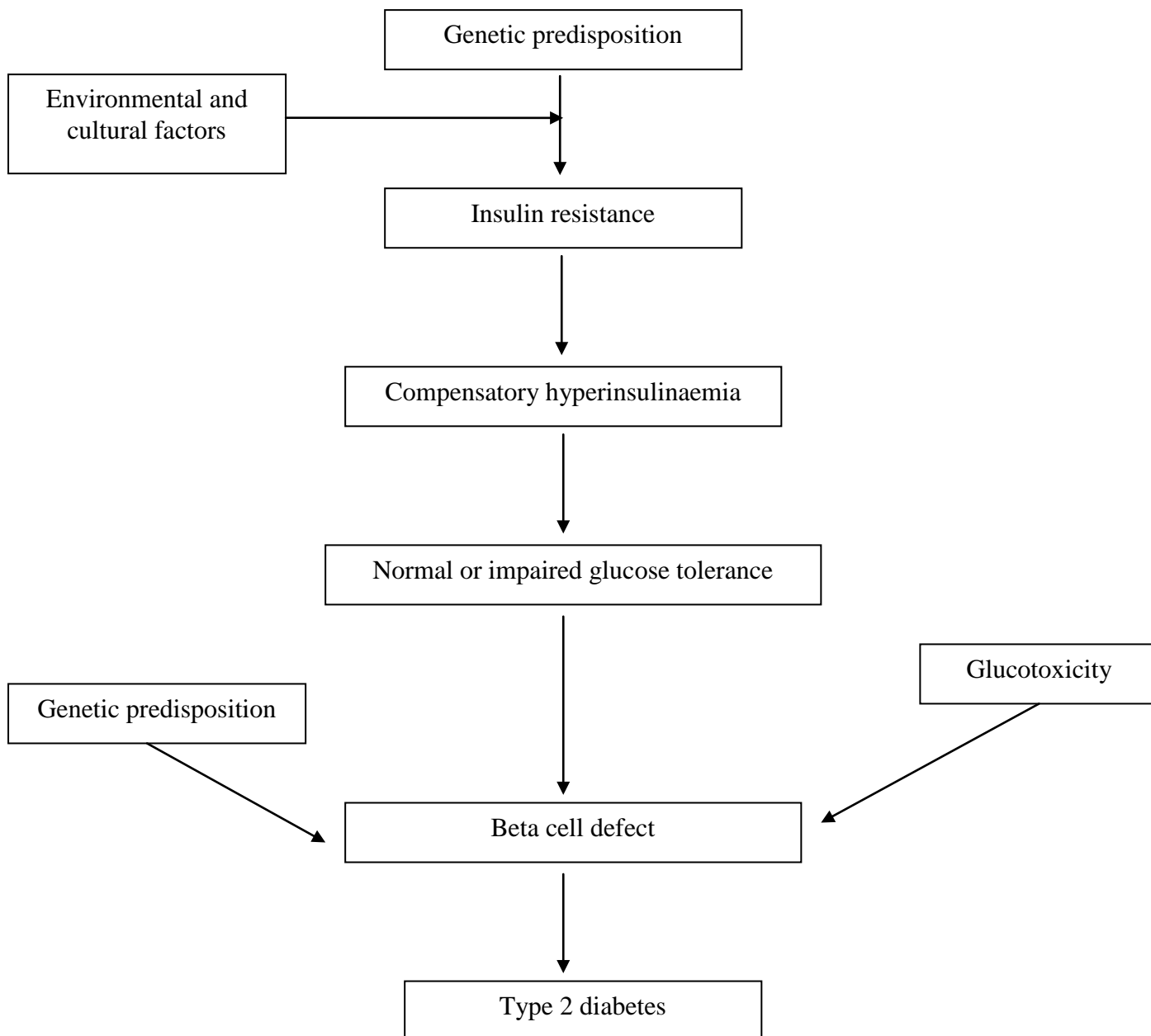
Diagram 2.1 The sequence of events leading to the diagnosis of type II diabetes mellitus (McDowell *et al*., 2007).

According to the report of the expert committee on the diagnosis and classification of diabetes mellitus (WHO, 2002), there are three ways/ criteria to diagnose diabetes and stage must be confirmed. The first criterion looks at symptoms of diabetes and cause (defined as any time of the day without regard to time since last meal) plasma glucose concentration $\geq$ 200 mg/dl, the second looks at fasting plasma

glucose (FPG) which must be ≥ 126 mg/dl (Table 2.2i) and the last criteria look at the two hour plasma (PG) ≥ 200 mg/dl during an oral glucose tolerance test (OGTT) (WHO, 2002) (Table 2.2ii). The clinician should take into consideration such additional factors as family history, age, adiposity and concomitant disorder before deciding on a diagnostic or therapeutic course of action (Alberti, et al, 1998). If the FPG and OGT test are positive, the test must be reconfirmed on a different day by repeating the both tests.

Table 2.2 (i) fasting plasma glucose test and (ii) oral glucose test

(i) Plasma glucose results (mg/dL)	Diagnosis
99 or below	Normal
100 to 125	Pre-diabetes (impaired fasting glucose)
126 or above	Diabetes

(ii) Two hour plasma glucose result (mg/dL)	Diagnosis
139 or below	Normal
140 to 199	Pre-diabetes (impaired glucose tolerance)
200 or above	Diabetes

2.4 Treatment

2.4.1 Insulin therapy

The main treatment of type I and more severe forms of type II diabetes mellitus is still dependent on insulin treatment. At present a number of different formulations of insulin are available for use in people. The differences are due to dose, source (animal or transgenic), purity and type (base on formulation) (rapid acting type e.g. "Toronto" insulin and slow/ intermediate type e.g. protamine zinc insulin) (Krall and Beaser, 1989). In addition from a clinical perspective, the formulation may be characterized by the onset of action (how quickly it works), time of peak activity (time to best effect) and duration of action. Irrespective of the type of insulin in use,

the aim of insulin therapy for patients with type I diabetes is to achieve normal glycaemia levels with minimal pathophysiological side effects (Davidson, 1991).

While the advent of modern insulin formulations has changed the lives of many diabetic suffers for the better, this has come with the occurrence of numerous adverse drug reactions:

2.4.1.1 Delayed local reaction at site of injection; the earlier impure preparations of insulin caused lesions at the site of injection mainly due to the impurities of the insulin preparations (Davidson, 1991).

2.4.1.2 Insulin allergy; true allergy to insulin also called systemic insulin allergy is rare and occurs in less than 0.1% of patients receiving insulin therapy. It is common in patients with a history of interrupted insulin therapy. The side effect beings as an immediate reaction at the injection site and spreads to the rest of the body in the form of a rash and may also cause oedema and anaphylactic shock (Davidson, 1991).

2.4.1.3 Insulin resistance; insulin resistance is defined clinically as a situation where the potent requires more than 200 IU of insulin daily for more than two consecutive days. Most commonly this condition may be associated with an increased incidence in infections and gross obesity (Davidson, 1991).

2.4.1.4 Insulin induced lipoatrophy; this side effect causes the loss of subcutaneous fat at the site of insulin injection. This is more common in young females but it is not limited to insulin dependent patients (Davidson, 1991).

2.4.1.5 Insulin induced lipohypertrophy; the condition is due to a local lipogenic effect of insulin and the use of pure insulin at the affected sites i.e. there's an accumulation of subcutaneous fat at this site. The continual injection of insulin at one site also results in diminished central effect as the subcutaneous fat accumulates at the site of injection. Injection in these areas should be avoided (Davidson, 1991).

2.4.2 Other forms of diabetic therapy

Other treatment available for use have become known as the oral hypoglycaemic as they can be administered orally unlike parenteral insulin. As the name implies, the oral hypoglycemic agents work by lowering the glucose levels in he blood. There are several antidiabetic medications available and their use depends on the nature of the diabetes, age, situation of the person and several other factors.

The most common forms of type II diabetic drugs are:

2.4.2.1 Sulphonylurea e.g. glibenclamide, are established oral hypoglycaemic agents that act by stimulating insulin secretion by the pancreatic β-cells. These drugs effectively reduce blood glucose levels in type II diabetic patients in the short term. These drugs have, however, not proved to be of much benefit in reducing the long term complications of the disease and at times may be associated with increased with weight gains may eventually lead to hypertension (Hanefeld, 1998).

2.4.2.2 Metfomin is a relatively old antidiabetic drug that peripheral insulin effect at the musculature and inhibits hepatic gluconeogenesis by enhancing the uptake of glucose into the peripheral cells. It does have a number of undesired side effects such as hepatic impairment, rnal impairment and heart failure and it has an anorexic effect (Hanefeld, 1998).

2.4.2.3 Acarbose is a novel antidiabetic drug that attenuates postprandial hyperglycemia by delaying carbohydrate digestion, without causing major side effects. Acarbose exert its inhibitory effects on the alph-glucosidases, a family of membrane bound enzymes in the intestine that are involved in the digestion and uptake of carbohydrate into the blood stream. Acarbose initiates a cascade of events which leads to improved metabolic control in type II diabetes of all stages, by stimulating both the synthesis and secretion of insulin and in addition improves glycemic control when administered concurrently with other antidiabetic agents. The most common side effect reported of acarbose therapy is meteorism and flatulence. There are rare cases of patients experiencing diarrhea resulting from the fermentation in the colon (Hanefeld, 1998).

2.4.2.4 Other therapies with a yet to be identified mechanism of effect are the plant extracts currently in use for the treatment and control both diabetes type 1 and 2 e.g. *Sutherlandia frutescens*, *H. hemerocallidea* and *Psidium guajava*.

2.4.3 Concurrent non-drug therapy

In addition to drug therapy, a proper diet is critical in the therapy of diabetes mellitus. Such diets should be balance and nutritious, ensuring normal growth and development and the attainment of the ideal body weight. When these diets are formulated, the total caloric intake of the patient is designed specially for the ideal weight of the patient. In addition these diets are designed so that the total caloric intake is divided between the categories of carbohydrates, protein and fat with the use of natural foods high in fiber being highly encouraged (McDowell *et al*, 2007).

Exercise in type II DM patients is usually beneficial as it is increases total energy expenditure, which when combined with a healthy diet, should assist in weight loss. Regular exercise also helps to maintain lean body mass (McDowell *et al*, 2007). However, in insulin dependent diabetic patients exercise may cause hypoglycaemia due to increased absorption of insulin from the injection site and enhanced effectiveness at the tissue level (Davidson, 1991).

2.5 Complication of diabetes

The most common complications of diabetes according to the American Diabetes Association (Touchette, 2005) are:

2.5.1 Cardiovascular disease; several cardiovascular disease occur and they are all due to problems in how the heart pumps blood or how the blood cieculates throughout the body. Diabetes medication may cause certain chemical changes in some of the substances found in the blood and this may lead to blood vessels narrowing or clogging up completely, resulting in a condition known as atherosclerosis. Hypertension is also a contributor of cardiovascular disease associated with diabetes (Touchette, 2005).

2.5.2 Retinopathy (cataracts); this is a very common complication of diabetes which affects the retina and more commonly affects type I diabetic patients. Two types are common, the nonproliferative type where the blood vessels are closed off or weaken in the eye and this leads to blurred vision without blindness. The proliferative type causes a proliferation or sprouting vessels in the retina which may lead to severe eye problem which may result in cataract formation (Touchette, 2005).

2.5.3 Nephropathy; in the people with this condition the nephrons are unable to filter out the impurities in the blood and these being to leak and impurities that are supposed to be removed from the body end up re-circulating in the blood. Not everyone with diabetes develops this condition and it is more common in type I diabetic patients than in type II diabetic patients (Touchette, 2005).

2.5.4 Neuropathy; while diabetes does not impair the brain or spinal cord, the peripheral nerves in the rest of the body is affected. This may lead to signal transduction errors which are interpreted aberrantly as pain in hands and feet or as a loss of sensation. This is not a prolonged condition and occurs for short periods of time. Several types of neuropathies occurs and the treatment varies according to the type; Distal symmetric polyneuropathy, focal neuropathy and autonomic neuropathy.

The later type occurs in 20 to 40% of people with long standing diabetes (Touchette, 2005).

2.5.5 Secondary infections; bladder infections caused by neuropathy, gingival infections and periodontitis, influenza, pneumonia, foot problems and in some women vaginal disease may occur (Touchette, 2005).

2.6 Herbal remedies and diabetes mellitus

The use of herbal remedies and plant derivatives has gathered a lot of interest since the 1980s and the use of complementary and alternative medicine has grown in many countries around the world. Studies conducted in several developed countries such as Australia, United Kingdom and United States of America report that almost half to two thirds of the population affected with diabetes use complementary and alterative medicine to control the condition (Ceylen *et al*., 2008). The use of herbal remedies and plant derivatives to help in the treatment of diabetes should certainly not be discounted. Although numerous "miracle herbal cure" companies exist, and champion the ability of herbal compounds to supplement insulin as a treatment, these should not be taken at face value without thorough research (Ceylan *et al*., 2008). A review of literature covering the area of diabetes research revealed many procedures used by researchers to investigate the hypoglycaemic effects of medicinal plants. Each procedure takes into account the aim and objectives of the study, thus a generalized schematic procedure has been summarized in Figure 2.3 to show the overview of the steps taken when investigating hypoglycaemic effects of medicinal plants (Heinrich *et al*., 2004; Harbone, 1998; Kinghom and Balandrin, 1993).

There has been a lot of success with the use of plant species to treat and control diabetes and its complications. Much of the success has only been observed experimentally in animal models. However, only a small number of these plants have been studied to evaluate the effect of the herbal therapies on their diabetic condition being managed. In many cases it has been observed and documented that the plants under study have the ability to regulate or contribute to the regulation and production of insulin and glucose to a degree (Ceylan *et al*., 2008). Sepici *et al*. (2004) conducted experiments on alloxan-diabetic rabbits and found that the oil from the myrtle plant (*Myrtus communis*) exerts effectual hypoglycaemic activity in the dicbetic anmal models used without inducing any toxicity.

Alcoholic stem extracts of *Coscinium fenestratum* lowered blood glucose levels in diabetic rats with no toxic effects observed (Shirwaikar *et al*., 2005).

Ojewole and Adewunmi (2004) tested the effect of Tetrapleura tetraptera fruit aqueous extracts in rats and found it to be effective in the control of adult onset diabetes (type II diabetes). Dimo *et al*., 2007 investigated the activity of Sclerocarya birrea extract in streptozotocin-induced rats and found that the extracts were able to decrease blood glucose and plasma insulin levels. More recently van de Venter *et al* (2008) performed *in vitro* studies on 11 plant species including *S. birrea*, previously shown to have *in vivo* antidiabetic activity, comfirming the *in vivo* work undertaken previously.

Wang *et al*. (2007) demonstrated that *P. guajava* extracts had significant inhibition of α-glucosidase activity in the small intestines of diabetic mice while a study by van de Venter *et al*. (2008) confirmed the inhibitory activity *in vitro*. *In vitro* assays on *Euclea undulate* and *Schkuhria pinnata* according to Deutschlander *et al* (2009) showed potential in lowering blood glucose levels in C2C12 myocytes cell lines at 50 μg/ml. The *in vitro* assay results from the same study also had α-glucosidase and α-amylase inhibitory activity of *Pteronia divaricata* and *Euclea undulate* at 50 μg/ml with IC_{50} values of 31.22 ± 0.154 and 49.95 ± 0.007 μg/ml respectively.
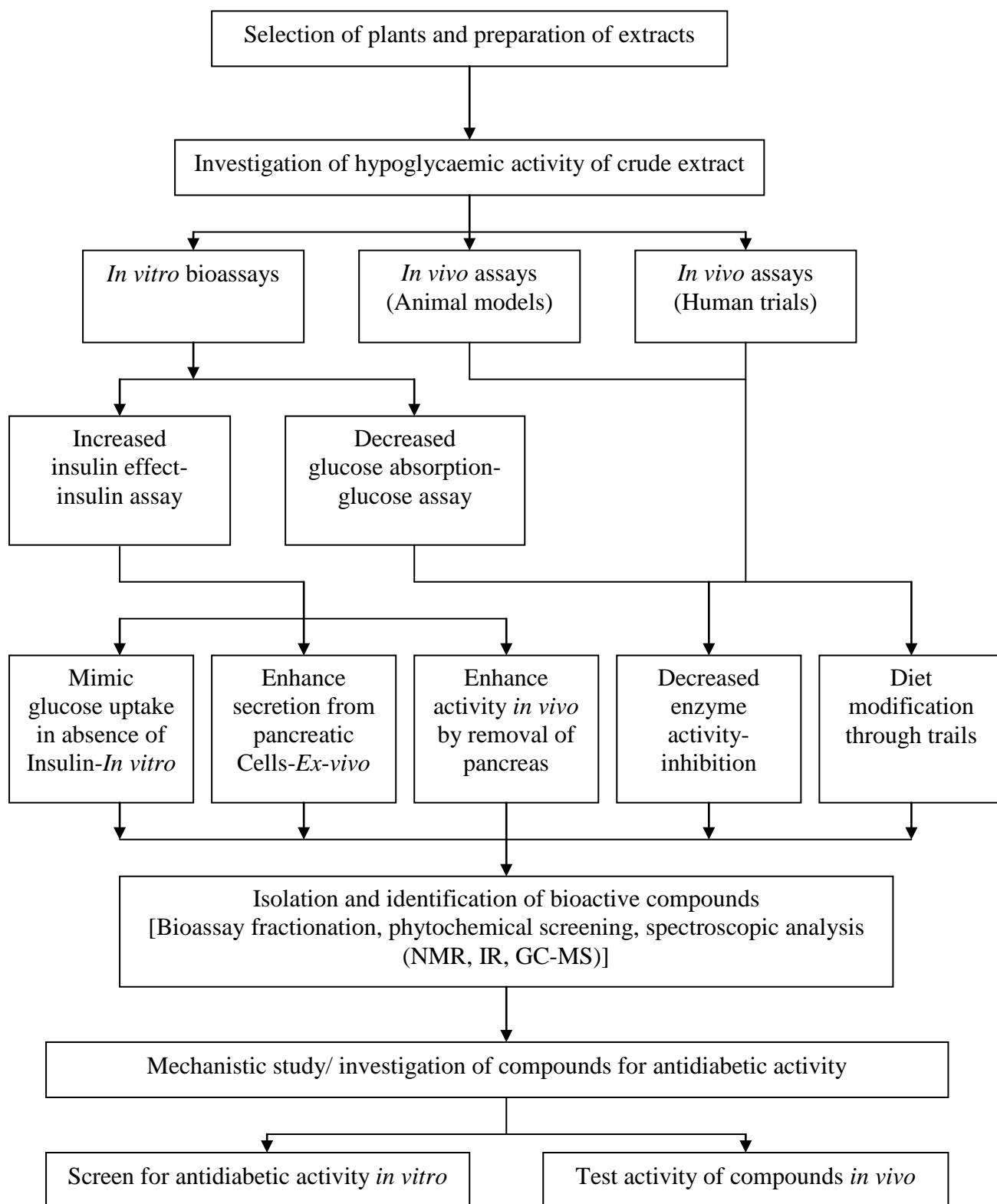
Figure 2.3 Schematic diagram for the investigation of antidiabetic/ hypoglycaemic activity in medicinal plants.

2.7 α-Glucosidase inhibitors

2.7.1 Definition

α-glucosidase inhibitors are inhibitors of α-glucosidase enzyme, which can benefit people with diabetes mellitus, work by reducing the amount of glucose that the intestines absorb from food. Normally, carbohydrates are converted into simple sugars (monosaccharides), which can be absorbed through the intestine within digestion activity of α-glucosidase emzyme. α-glucosidase inhibitors inhibit α-glucosidase emzyme system reduces the rate of digestion of carbohydrates. Result that less glucose is produced because the carbohydrates are not broken down into glucose molecules. The benefit to the diabetic patients is to decrease current blood glucose levels. In natural source there are many types of glucosidase inhibitors both sugar form and non-sugar form. Sugar or sugar derivatives such as disaccharides, immunosugar, carbasugars, thiosugars and glycosides (Melo *et al.*, 2006). Non-sugar derivatives such as protein and glycoprotein (lectin).

2.7.2 Clinical application

α-glucosidase inhibitors as a medicine, starch blockers help control blood sugars by slowing down the digestion of complex carbohydrates, for diabetes mellitus such as Acarbose (Precose), a prominent α-glucosidase inhibitor, was first isolated from soil bateria *Actinoplanes* sp (Melo *et al.*, 2006), Miglitol (Glyset) was discovered as the most favorable inhibitor of *in vivo* active agents which showed reducing postprandial blood glucose level and Voglibose, which has high inhibitory activity against sucrase and maltase, has been employed in Japan for the treatment of diabetes since 1994. It was synthesized from valiolamine via reductive amination with dihydroxy acetone. In current studies based on α-glucosidase inhibitory activity, it was shown to be 20 to 30 times more potent than acarbose. In 1996, 1-deoxynojirimycin was isolated from the roots of mulberry tree. Despite the excellent α-glucosidase inhibitory activity in vitro, its efficacy in vivo was only modulate (Melo *et al.*, 2006).

2.7.3 Literature reviews of α-glucosidase inhibitors

Chiasson *et al.* measured the effects of the chronic use of the α-glucosidase inhibitor acarbose over a 3 year period; Refer to Figure 2.4. Subjects with impaired glucose tolerance were allocated into two groups: 71 were placed in the acarbose group and 715 were placed in the placebo group. The acarbose and placebo were

taken 3 times a day for 3.3 years by the respective groups, immediately before beginning consumption of a meal. Chiasson *et al.* found a reduced risk of progression from impaired glucose tolerance to diabetes of 25% in the acarbose group compared with the placebo group. This reduction in risk was hypothesized to occur because of the decreased postprandial glucose response due to the blocked α-glucosidase enzyme by acarbose. Chiasson *et al.* also hypothesized that the increased reversion to normal glucose tolerance in the acarbose group was due to the decreased postpradial glucose response as well.
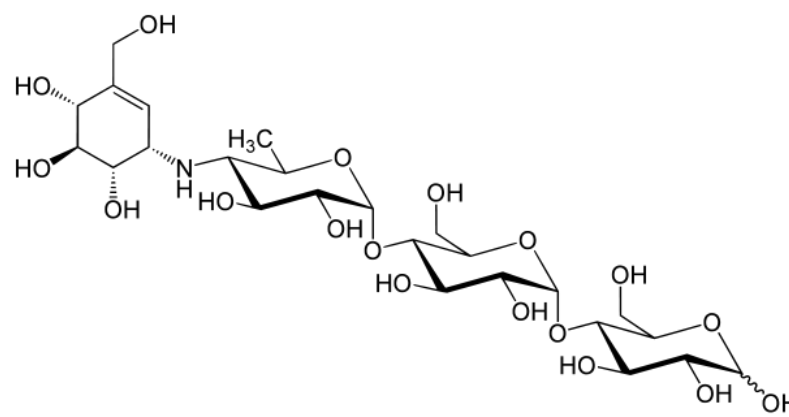
Figure 2.4 Structure of Acarbose. Acarbose mimics the oligosaccharide substrate and binds to α-glucosidase with a greater binding affinity than oligosaccharides.

Hanefeld *et al.* conducted a meta-analysis of seven randomized, placebo-controlled acarbose studies with a minimum duration of 52 weeks. All patients in these studies had diabetes, with the vest majority being type 2 diabetics. Under acarbose treatment, a significant prolongation of time in which patients remained free of any newly diagnosed cardiovascular events occurred compared with patients under the placebo treatment. Additionally, a 64% relative risk reduction for myocardial infarctions was observed for the acarbose for the acarbose treatment group. Long term glycemic control was observed in the acarbose treatment group compared with the placebo group, with a significant reduction in HbA1C levels, fasting and postpradial blood glucose levels in acarbose patients. Patients in the acarbose group also demonstrated lowered plasma triglycerides levels, body weight, BMI, systolic blood

pressure, reduced insulin levels, and increased insulin sensitivity secondary to reduced postprandial glycemia.

Extracts from the roots and stems of *Salacia oblonga*, a woody climbing plant that grows in parts of India and Sri Lanka, have been shown to have α-glucosidase inhibitory activity *in vitro* and may be useful in the prevention and/ or treatment of diabetes. Two thiosugars isolated from *S. oblonga* extract, salacinol and kotalanol, have inhibitory effects, *in vitro*, against maltase, isomaltase and sucrase with the inhibitory effect against sucrase being more potent than the prescription α-glucosidase inhibitors acarbose and voglibose that are used in the treatment of diabetes. Through the reduction of the enzymatic breakdown of di-, tri-, and oligosaccharides by α-glucosidase, carbohydrate absorption is decreased, attenuating the postprandial glycemic response. The undigested di-, tri-, and oligosaccharides pass through the small intestine into the colon where they are digested by the colonic microflora producing gaseous by products, demonstrated by the increased breath hydrogen responses in Heacock *et al*. Lowering of postprandial glycemia by *S. oblonga* extract has been observed in rats fed either maltose or sucrose, but not glucose, which is consistent with its α-glucosidase inhibitory effect in the intestine.

Salacinol contains a zwitterions consisting of a sulfonium ion with an internal sulfate counterion. It is hypothesized that the permanent positive charge on the sulfur atom in the 1,4-anhydro-4-thio-D-arabinitol moiety binds to α-glucosidase through mimicry of the shape and charge of the oxacarbenium-ion intermediate in the hydrolysis reaction mediated by α-glucosidase. Kotalanol contains the same 1,4-anhydro-4-thio-D-arabinitol moiety and is believed to work via the same mechanism as salacinol.
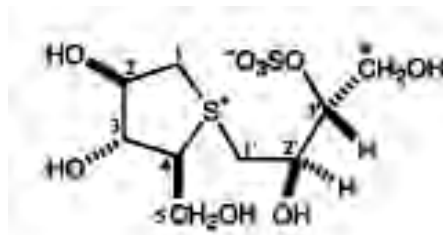
Salacinol
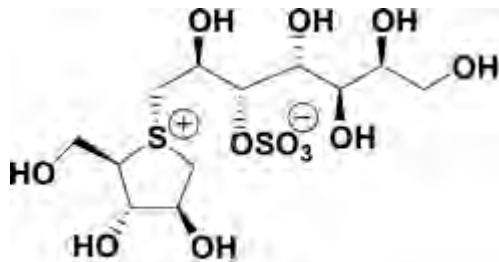
Kotalanol

Figure 2.5 Structure of salacinol and kotalanol. The permanent positive charge residing on the sulfur atom in the 1,4-anhydro-4-thio-D-arabinitol moiety may be responsible for the alpha-glucosidase inhibition in vitro.

Heacock *et al*. and Collene *et al*. have demonstrated that S. oblonga extract reduces postprandial glycemia and insulinemia when it is fed in addition to a liquid nutritional supplement containing mainly maltodextrin (61% of available carbohydrate) as the carbohydrate source. Heacock *et al*. measured the effects of varying doses of *S. oblonga* extract on postprandial glycemia in 39 healthy subjects. Subjects were fed a test beverage containing 49.5 g maltodextrin with and ithout 1000 mg of *S. oblonga* extract. Serum glucose AUC was reduced by 23% and serum insulin AUC was reduced by 29% to the test beverage containing 1000 mg of extract compared with the test beverage alone. An increased breath hydrogen response was also measured with the 1000 mg treatment, indicating that carbohydrate malabsorption was at least partially responsible for the decreased glycemic response. These results are consistent with previous studies measuring postprandial glycemia using acarbose.

Collene *et al*. measured *S. oblonga* extract and free amino acids on postprandial glycemia in 43 healthy subjects. This study found a 24% decrease in

postprandial AUC glycemic response to a test beverage containing 82 g carbohydrate, 20 g protein, and 14 g fat with 1000 mg *S. oblonga* extract compared with the test beverage alone. Serum insulin response to the test beverage was also lower with the addition of *S. oblonga*. Extract fed with the beverage in addition to 3.5 g of phenylalanine and leucine led to a 27% decrease in postprandial AUC glycemia compared with the test beverage alone. Serum insulin response to the extract plus free amino acid meal was insignificantly lower compared with the serum insulin response to control.

Other studies have determined that chronic use (daily use for 2-3 months) of *Salacia* herbs might also improve long-term glucose control. Jayawardena *et al*. tested *S. reticulat*a use for 3 months on 51 subjects with type 2 diabetes mellitus. *S. reticulata* also contains the α-glucodisae inhibitors salacinol and kotalanol. Subjects in this study consumed Kothala Himbutu tea daily for six months: three months of which contained *S. reticulate* extract and three months of which did not. A decrease in HbA1C was observed in patients after the *S. reticulate* treatment than after the placebo treatment (6.29 +/- 1.02 in treatment vs. 6.65 +/- 1.04 in placebo). Jayawardena et al. also reported no significant abnormalities in liver or renal function after treatment with *S. reticulate*, with the main effect reported being loose stools.

The safety profile of *Salacia* herbs has been studies in laboratory animals. Wolf and Weisbrode measured the effects of *S. oblonga* extract in a two week trial using Sprague-Dawley rats at a dose approximately 10 times higher than used in human trials. The rats fed the extract showed significantly reduced weight gain and relative liver and spleen weights; however no significant histopathological changes in hepatic or renal functions occurred. Shimoda *et al*. also showed no adverse effects on food intake, body weight, blood chemistries, organ weights, or histopathological findings on rats fed *S. reticulata* at doses up to 1000 mg/kg for 13 weeks of continuous intake, Results observed by Ratnasooriya *et al*., however, show that *S. reticulat*a fed to Wistar rats during early to mid-pregnancy was associated with increased post-implantations losses, reduced birth weight of the pups, reduced fetal survival ratio, and reduced viability ratio at a dose 170 times greater than doses fed previously in humans.

In 2001 Lee *et al*., had a research of isolation and identification of genistein as a candidate for α-glucosidase inhibitor from fermentation broths of a *Streptomyces* sp.

In 2006, the inhibitory activity of six group of flavonoids against yeast and rat small intestinal α-glucosidase and porcine pancreatic α-amylase was compared. Yeast α-glucosidase was potently inhibited by the anthocyanidin, isoflavone and flavonol groups with the IC$_{50}$ values les than 15 µM (Tadera *et al*., 2006).

In 2007 Jong-Anurakkul *et al*., had a research of isolation of α-glucosidase inhibitors from 24 traditional Thai medicinal plant samples. Potent alpha-glucosidase inhibitory activity was found in aqueous methanol extract of Devil tree, *Alstonia scholaris*, leaves. Active principles against alpha-glucosidase, prepared from rat small intestine acetone powder, were isolated and identified. The structures of these isolated compounds were found to be quercetin 3-O-β-D-xylopyranosyl (1'''⟶ 2'')-β-D-galactopyranoside and (-)-lyoniresinol 3-O-β-D-glucopyranoside on the basis of chemical and spectral evidence. The latter exhibited an inhibitory activity against both sucrase and maltase with IC$_{50}$ values of 1.95 and 1.43 mM, respectively, whereas the former inhibited only maltase with IC$_{50}$ values of 1.96 mM.

In 2008, Lam *et al*., had a research of bioassay-guided fractionation against α-glucosidase resulted in isolation and characterization of eight active compounds from the EtOH extract of the seeds of *Syagrus romanzoffiana* (Lam *et al*., 2008). Besides that, Lee *et al*., had found bioassay-guided fractionation against α-glucosidase from the leaf extract of *Machilus philippinese* Merr (Lee *et al*., 2008).

2.7.4 Purification technique of protein

The protein was purified from Crude extract of mixer solution, commonly use chromatography technique such as, affinity chromatography, ion exchange chromatography, and gel filtration chromatography. In 2004 had a research that used affinity chromatography to purify the lectin from human serum proteins by Con A sepharose column coupled to two-dimensional gel electrophoresis. The purified sample had 2 fractions before use this technique. (Rodriguez-Pineiro *et al*., 2004). Next year, a lectin from the marine red alga *Gracilaria ornata* (*Gracilariaceae, Rodophyta*); GOL was purified by 2 steps chromatography technique consist of ion exchange chromatography on DEAE-cellulose and affinity chromatography on mucin-Sepharose 4B. The GOL significantly affected the development of *Callosobruchus maculatus* larvae, indicating the possibility of using this lectin in a

biotechnological strategy for insect management of stored cowpea seeds. (Leite *et al.*, 2005). In 2007 Shi *et al.* study lectin from raw and canned red kidney bean (*Phaseolus vulgaris*). They used gel filtration technique to purify. Use Affi-gel Blue gel sepharose compare to thyroglobulin-Sepharose to purify the lectin from red kidney bean. Found that the lectin from thyroglobulin more purify than Affi-gel Blue gel. (Shi *et al.*, 2007)

2.8 *Archidendron jiringa* Nielsen.

2.8.1 General Background

2.8.1.1 Classification

Family: Leguminosae

Genus: *Archidendron*

Species: *Jiringa*

Common manes: Jiringa or Djenkol bean

2.8.1.2 Description

Archidendron jiringa Nielsen. is belonging to Leguminosae family. Its common name is jiringa or djenkol bean. This legume is a perennial plant that is native to Southeast Asia with the approximately 10-15 meter height. Its bark is gray or brown-gray. The leaves are tapering and the flowers are white and small size. This tree produces twisted brown seed pods containing individual seeds that are approximately 4-5 cm in size. This legume is very common in the secondary forests of our Nature reserves and Nature Parks, and most likely this tree was cultivated and its fruit is used in the preparation of salads. The seeds exhibit a very strong garlic smell. Its beans are a popular food in Indonesia, and are also consumed in Malaysia (where they are known as *jering*), Myanmar (where they are called da nyin thee) and in Southern Thailand, where it is called luk neang. Its beans are very popular fresh eat with chili paste in Southern Thailand.

The beans are mildly toxic due to the presence of djenkolic acid, an amino acid, which causes djenkolism (jengkol bean poisoning). It causes "spasmodic pain, gout, urinary obstruction and acute renal failure". The condition mainly affects men, and is not determined by how the beans are prepared, and individuals can consume the beans on multiple occasions without incident, to develop renal failure on another occasion

Figure 2.6 *Archidendron jiringa* Nielsen seeds.

2.8.1.3 Literature reviews of Leguminosae family

Shi *et al*. (2007) have a research of The bioactive properties of lectins obtained from raw and canned red kidney bean (*Phaseolus vulgaris*) were studied to determine the changes in their bioactivity during the canning process. Phytohaemagglutinin (PHA) was extracted using Affi-gel Blue gel and thyroglobulin-Sepharose and had a molecular weight of 32 kDa. Both the raw and the canned kidney beans possessed the ability to agglutinate red blood cells and inhibit aglucosidase. The activity found in the canned beans was similar to that from the in the raw kidney beans. However, the amount of lectin that could be extracted from thyroglobulin-Sepharose was much less in the canned samples than in the raw kidney bean samples. The extracted lectin from the raw kidney beans was also subjected to a heating and cooling treatment using a differential scanning calorimeter. The lectin had a nonset denaturation temperature of 77.76 8C and it did not renature upon cooling. In the study, they demonstrated that extracts from raw red kidney bean and canned red kidney bean contain bioactive compounds capable of inhibiting HIV-1 RT in vitro (Shi *et al*., 2007).

Boonmee *et al*. (2007) have a research of two alpha-glucosidase inhibitors which were isolated from the flower of *Sesbania grandiflora* and named SGF60 and

SGF90. The procedure involed extraction with phosphate buffer, precipitation with ammonium sulfate, ion-exchange chromatography on DEAE-cellulose and gel filtration on Superdex-200. These protein were identified by using tandem mass spectrometry. The results show partial amino acid sequence of SGF60 similar to p27SJ, a protein from *Hypericum perforatum* found to suppress HIV-1 gene expression. SGF90 matched a beta-glucosidase from *Arabidopsis thaliana* (Boonmee *et al.*, 2007).

Keyaerts *et al.* (2006) had a research of *Parkia pendula* seed lectin effect for Human cytomegalovirus (HCMV) and human herpes virus 6(HHV-6). Found that HCMV *in vitro* infectivity was inhibited but in contrast to HHV-6 which was not affected. (Favacho *et al.*, 2007). A research of effect of various plant lectin to corona viruses, namely the SARS-CoV (severe acute respiratory syndrome) and FIPV (feline infectious peritonitis virus). Found that 15 lectins had antiviral properties against both Coronaviruses; 5 plant lectins were active only against SARS-CoV and 2 lectins showed solely activity against FIPV. Eight lectins were inactive against both SARS-CoV and FIPV. And *Hippeastrum hybrid* agglutinin (HHA) had a good anti-coronarirus activity (Keyaerts *et al.*, 2007).

CHAPTER lII

EXPERIMENTAL

3. Material and methods

3.1. Plant materials

The fresh seeds of *Archidendron jiringa* were purchased from the local market in Bangkok, Thailand.

3.2 Chemicals and reagents

ConA Sepharose was purchased from Sigma Chemicals Co. (USA). Methyl-α-D-glucopyranoside and EDTA were purchased from Fluka (Germany). Bovine serum albumin, *p*-nitrophenyl α-D-glucopyranoside*, p*-nitrophenol, and α-glucosidase, Type l: from bakers yeast were the product of Sigma Chemicals Co. (St. Louis, MO, USA). Potassium hydrogen phosphate (KH_2PO_4), dipotassium hydrogen phosphate (K_2HPO_4), sodium chloride (NaCl), sodium carbonate (Na_2CO_3), sodium hydroxide (NaOH), were obtained from Merck (Germany). Magnesium chloride, manganese chloride and copper sulfate, were obtained from Fluka (Switzerland). Tris was purchased from USB (USA). Calcium chloride, ferric chloride, zinc sulfate, methanol, ethanol, sodium acetate and acetic acid were purchased from Merck (Germany). Double distilled water was used in this research was prepared with glass water stills (GFL Gesellschaft fur labortecilk mbH, Germany). All other biochemicals and chemicals used in the investigation were of analytical grade.

3.3 Apparatus and instruments

Liquid chromatography run on AKTA prime (Amersham pharmacia biotech, wikstroms, Sweden), Micropipette (Pipetteman, Gilson, France), Vortex mixer (Vortex-genie 2, Sciencetific Industries, U.S.A.), pH meter (Denver Instrument U.S.A. system), Sonicate (DHA-1000, Branson, U.S.A.), Orbital Shaker (Kika-Werke GMBH&Co., Germany), Refrigerated centrifuge (Himac CR20B2, HHITACHI, Japan), Water Bath Shaking (Memmert, Germany), Power supply (EPS 3500 XL, Pharmacia, England), Dialysis bag (Rockford, U.S.A.), Freeze dryer (Laboconco, U.S.A.), Microcentrifuge (Biofuge pico Heraeus, Kendro, Germany), and

Spectrophotometer (TECAN, Astria), Surface Plasmon Resonance (SPR) Analyzer (Autolab ESPRIT, Eco Chemie B.V., Utrecht, The Natherlands).

3.4 Extraction of protein from seeds of *A. jiringa*

One kilogram of *A. jiringa* seeds was homogenated and, defatted with acetone at 4 ^{o}C (200 ml aqueous acetone per 10 g seed). Remove the insoluble material by vacuum filtration and then extracted overnight at 4 ^{o}C with 20 volumes of TBS (Tris-Buffered Saline, 20 mM Tris-HCl, pH 7.2 plus 150 mM NaCl, w/v). The suspension was then clarified by filtration through a double-layered cheesecloth followed by centrifugation at 15,000 × g for 30 min. The clarified supernatant was then harvested and ammonium sulfate added, with stirring, to 90% saturation and then left with stirring overnight at 4 ^{o}C. The precipitate was collected from the suspension by centrifugation at 15,000 × g for 30 min with discarding of the supernatant. The pelleted material was then dissolved in TBS, dialyzed against excess water and then freeze dried.

3.5 Purification of protein from seeds of *A. jiringa*

ConA Sepharose was pre-equilibrated with TBS and transferred to a 1.6 × 20 cm column. The 90% saturation ammonium sulphate precipitated seeds extract was applied to the column (10 ml at a total protein concentration of 2 mg/ml) at a flow rate of 1.5 ml/min, and the column was then washed with TBS at the same flow rate, collecting 10 ml fractions, until the A_{280} fell to <0.05. The bound proteins, including lectins, were then eluted from the column, using TBS supplemented with 0.2 M methyl-α-D-glucopyranoside as the competitor, at the same flow rate and collecting 10 ml fractions. The fractions containing lectin, on the basis of A_{280} and a detectable hemagglutination activity against rabbit erythrocytes were combined, dialyzed against excess water, frozen and lyophilized.

3.6 Protein concentration

The protein content was determined by Bradford's procedure (Bradford, 1976). Bovine serum albumin (BSA) was used as the standard with four different concentrations between 5 - 20 µg/ml to construct the calibration curve. Each sample was serially two-fold diluted with deionized water and then 50 µl aliquots of each dilution were transferred into each well of a microtiter plate and 50 µl of Bradford's reagent added to each well. The plate was shaken for 5 min and then left for 10 min before reading the absorbance at 595 nm using an ELISA plate reader. The obtained

OD was calculated for the protein concentration using the linear equation computed from the standard curve. During the column chromatographic separations, the elution peak profiles of proteins were determined by measuring the absorbance at 280 nm.

3.7 Carbohydrate determination

The phenol-sulfuric acid technique was slightly modified from the reported procedure (Dubois *et al*., 1956), by scaling up and using glucose as the standard. The enriched AI protein fraction (post Superdex-75 gel chromatography) was serially diluted and 500 μl aliquots of each dilution was transferred into 15 ml glass tubes, to which 500 μl of a 4% (w/v) phenol solution was added, thoroughly mixed and then left at room temperature for 5 min. Next, 4 ml of conc. H_2SO_4 was added into each tube, carefully mixed using a vortex mixer and 100 μl aliquots transferred into the well of a microtitre plate and the absorbance read at 492 nm. The obtained data was used to calculate the sugar content (glucose equivalent) using the standard curve developed from five different concentrations of glucose (range 10 - 50 μg/ml) analyzed by the same procedure. Glucose (50 μg/ml) in deionized water and deionized water alone were used as the positive and negative controls, respectively, in the assay.

3.8 α-glucosidase inhibitory activity

α-glucosidase inhibitory activity was done at every step of isolation. α-glucosidase enzyme and *p*-nitrophenyl-α-D-glucopyranoside (PNPG) was dissolved in 20 mM phosphate buffer, pH 7.2. 20 μl of α-glucosidase (1U/ml) was mixed with 10 μl of protein sample and 60 μl of phosphate buffer, the mixer was incubated at 37 °C for 10 min. and 10 μl of 1 mM PNPG was added as a substrate. After incubated at 37 °C for 35 min stopped the reaction with 100 μl of 0.5 M Na_2CO_3. α-glucosidase inhibitory activity was determined by measuring the release of the yellow *p*-nitrophenol at 400 nm.

$$\% \text{ Inhibition} = \frac{(OD_{control}-OD_{blank})-(OD_{sample}-OD_{blank}) \times 100}{(OD_{control}-OD_{blank})}$$

3.9 IC$_{50}$ determination

The half maximal inhibition concentration (IC$_{50}$) determines the concentration of the protein sample that inhibited 50% of the maximal α-glucosidase enzyme activity. Determining by make the two-fold dilution of protein sample and follow the protocol of α-glucosidase inhibitory activity testing described in 3.8.

3.10 Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

The gel was prepared with 0.1% (w/v) SDS in 15% separating gels and 5% stacking gels. Tris-glycine buffer pH 8.3 containing 0.1% SDS was used as the electrode buffer. Discontinuous SDS-PAGE in reducing conditions was performed according to the procedure of Laemmli. (Laemmli, 1970) Samples to be analyzed were treated with reducing sample buffer and boiled for five min prior to application to the gel. Electrophoresis was performed at a constant current of 20 mA per slab at room temperature in a Mini-Gel Electrophoresis unit. Molecular weight standards were co-resolved in adjacent lanes and used to determine the subunit molecular weight of the purified protein(s). After electrophoresis, proteins in the gel were visualized by staining with Coomassie Brillion blue R-250, (CBB).

3.11 Effect of temperature on the α-glucosidase inhibitor activity and thermostability

The effect of temperature on the α-glucosidase inhibitor activity was determined by incubating the enriched α-glucosidase inhibitor protein fraction samples in 20 mM phosphate buffer, pH 7.2 at various temperatures (4 - 90 °C at 10 °C intervals) for 30 min. The thermostability of the α-glucosidase inhibitor was investigated by incubating the α-glucosidase inhibitor protein fraction sample at 70, 80 and 90 °C in 20 mM phosphate buffer, pH 7.2 for the indicated fixed time intervals (10 - 120 min), cooling to 4 °C and then assaying the residual α-glucosidase inhibitor activity with 100% and 0% activity controls, as described in section 3.8.

3.12 The pH-dependence of the α-glucosidase inhibitor activity

Incubating the enriched α-glucosidase inhibitor protein fraction samples in buffers of broadly similar salinity levels, but varying in pH from 2 - 14 was used to assess the pH stability and the pH optima of the AI. The buffers used were 20 mM glycine-HCl (pH 2 - 4), 20 mM sodium acetate (pH 4 - 6), 20 mM potassium phosphate (pH 6 - 8), 20 mM Tris-HCl (pH 8 - 10) and 20 mM glycine-NaOH (pH 10 - 12). The enriched α-glucosidase inhibitor protein fraction was mixed in each

of the different buffer-pH compositions, or 20 mM phosphate buffer, pH 7.2, and then left for 1 hour at room temperature. Next, the samples were adjusted back to 20 mM phosphate buffer, pH 7.2 and assayed for α-glucosidase inhibitor activity (section 3.8) and the activities attained were compared with the control which was set as 100% activity.

3.13 Effect of metal ions on the α-glucosidase inhibitor activity

The effect of preincubation of the enriched α-glucosidase inhibitor protein fraction with six different divalent metal cations on the resultant α-glucosidase inhibitor activity was evaluated as follows. The enriched α-glucosidase inhibitor protein fraction (1 mg / ml) was incubated for 10 h with one of Ca^{2+}, Co^{2+}, Fe^{2+}, Mg^{2+}, Mn^{2+}, and Zn^{2+} at one of the various concentrations in 20 mM phosphate buffer, pH 7.2 with continuous shaking and was then tested for α-glucosidase inhibitor activity as described in section 3.8 using at least three replicates for each assay.

3.14 Mechanism of the inhibition

To evaluate the inhibition mode of the enriched α-glucosidase inhibitor protein fraction sample against the activity of α-glucosidase, PNPG solution at one of 0.025 - 0.2 mM, as the substrate, was added to the α-glucosidase (1 U/ml) in 20 mM phosphate buffer, pH 7.2 in the presence of 0, 0.05 and 0.075 mg/ml of the enriched α-glucosidase inhibitor protein fraction sample. The remaining α-glucosidase activity was determined as outlined in section 3.5. The inhibition type was determined by Lineweaver-Burk plot, where v is the initial velocity and [S] is the substrate concentration used.

3.15 Internal amino acid sequence of α-glucosidase inhibitor by liquid chromatography/mass spectrometry/mass spectrometry (LC/MS/MS)

3.15.1. *In situ* (in gel) trypsinization

The sample preparation process followed the published method of Mortz *et al.* (1994). Each band in the electrophoretic gel was excised, cut into small pieces (ca. 1 mm^3) and washed with 100 μl deionized water. The gel pieces were destained by adding 200 μl of a 2:1 (v/v) ratio of acetonitrile: 25 mM NH_4HCO_3 for 15 min, and this step was performed several times until the gel pieces were completely destained. The supernatant was removed and gels were then dehydrated by adding 200 μl acetonitrile for 15 min prior to drying in a vacuum centrifuge. Then 50 μl of a 10 mM DTT solution in 100 mM NH_4HCO_3 was added, and the proteins were reduced for 1 h

at 56 ^{o}C. After cooling to room temperature, the DTT solution was replaced with the same volume of 55 mM iodoacetamide in 100 mM NH_4HCO_3 and the gels were incubated for 45 min at room temperature in the dark. The solution was then removed, the gel pieces were dehydrated in acetonitrile and the solvent evaporated off before adding 10 µl of a trypsin solution (proteomics grade, Sigma) (10 ng/µl in 50 mM NH_4HCO_3). After allowing the gel plug to swell for 15 min at 4 ^{o}C, 30 µl of 50 mM NH_4HCO_3 was added and the digestion proceeded at 37 ^{o}C overnight. The supernatant was then harvested following centrifugation at 15,000 × g for 1 min. The remaining peptides in the gel were extracted with a solution of 50% (v/v) acetonitrile containing 5% (v/v) formic acid for 10 min with shaking, and subsequently pooled with the supernatant and taken to dryness.

3.15.2 LC-MS/MS and peptide blasting

The likely amino acid sequence of each internal fragment of the trypsinized peptide was analyzed by LC/MS/MS mass spectrometry. The extracted tryptic peptides were then subjected to LC-nano ESI/MS/MS. All collected LC/MS/MS data were processed and submitted to a MASCOT (http://www.matrixscience.com) search of the NCBI database (http://blast.ncbi.nlm.nih.gov). The following criteria were used in the Mascot search: trypsin cleavage specificity with up to three missed cleavage sites, cysteine carbamidomethyl fixed modification, methionine oxidation variable modifications, ± 0.2 Da peptide tolerance and MS/MS tolerance, and ESI-TRAP fragmentation scoring (Mortz *et al*., 1994).

3.16 Biospecific interaction determination by Surface Plasmon Resonance (SPR)

Biospecific interaction was determined by Surface Plasmon Resonance (SPR) using Autolab ESPRIT system from Eco Chemie B.V., Utrecht, The Natherlands.

Surface Plasmon Resonance (SPR) is and optical technique based on the excitation of plasmons in a dielectric close to a metal surface. Excitation takes place by light from a laser under different incidence angle, at a specific angle, all the energy from the photons is transferred to the free electrons in the metal surface, which lead to total attenuation of this happens depends on the refractive index of the interface between metal surface and a dielectric (in this case is solution). The parameters that influence the refractive index are any change of mass on the metal surface, in other words the presence of biomolecules on or close to the metal surface.

Biospecific interactions, between α-glucosidase inhibitor from seed of *Archidendron jiringa* and alpha-glucosidase enzyme, studies were performed on Autolab ESPRIT system. Biosensor system based on the principle of surface plasmon resonance (SPR). The various concentration of protein sample in 20 mM phosphate buffer, pH 7.2 was coupled to a certified grade 11 MUA gold plate. The unreacted groups, on the suface of the gold, were blocked with ethanolamine. All measurements were analyzed by 1U/ml α-glucosidase enzyme.

CHAPTER IV

RESULT AND DISCUSSION

The treatment goal of diabetes patients is to maintain near normal levels of glycemic control, in both the fasting and post-prandial states. Many natural resources have been investigated with respect to suppression of glucose production from carbohydrates in the gut or glucose absorption from the intestine (Matsui *et al*., 2007). α-Amylase catalyses the hydrolysis of α-1,4-glucosidic linkages of starch, glycogen and various oligosaccharides and α-glucosidase further breaks down the disaccharides into simpler sugars, readily available for the intestinal absorption. The inhibition of their activity, in the digestive tract of humans, is considered to be effective to control diabetes by diminishing the absorption of glucose decomposed from starch by these enzymes (Hara and Honda, 1990). Therefore, effective and nontoxic inhibitors of α-amylase and α-glucosidase have long been sought. In addition, the alpha-glycosidase inhibitors have wide application for treatment of carbohydrate mediated diseases such as diabetes (Fujisawa *et al*., 1991), cancer (Humphries *et al*., 1986; Pili *et al*., 1995), (Shimizu *et al*., 1990), and certain forms of hyperlipoproteinemia and obesity (Sou *et al*., 2001). The α-glucosidase enzyme is required for the breakdown of carbohydrates to absorbable mono-saccharides at the intestine. The α-glucosidase inhibitor is usually used to prevent or medically treat type II diabetes (Non-insulin-dependent diabetes mellitus (NIDDM). These inhibitors combine with intestine alpha-glucosidase and block the uptake of postprandial blood glucose. (Holman, 1998)

4.1 Enrichment of the proteinaceous α-glucosidase inhibitor from the seeds of *A. jiringa*

The present report represents the first investigation on the purification of a lectin from *A. jiringa* seeds. *A. jiringa* seeds were homogenated and defated to form a crude soluble extract and, after 90% saturation ammonium sulphate precipitation and dialysis, the α-glucosidase inhibitor was purified in a single step by affinity chromatography column using ConA Sepharose, yielding a single apparent α-glucosidase inhibitor at ~3.3% (w/w) of the total starting seed weight. The initial 90% saturation ammonium sulfate precipitation resulted in an affinity chromatography

with a ConA Sepharose column resulted in two factions, an un-bound fraction that eluted with the TBS wash through and did not show any detectable alpha-glucosidase inhibitory activity, and a bound fraction that eluted with the presence of 0.2 M methyl-α-D-glucopyranoside and had alpha-glucosidase inhibitory activity with IC_{50} value of 0.031 ± 0.02 mg/ ml (Figure 4.1).

Affinity chromatography presents advantages in relation to other conventional methods due to its specificity and thus allows a reduced number of steps and gives high yields and purity (Goldenberg, 1989). As such it is widely used in the purification of glycoprotein. For example, the mannose-glucose specific lectins from the seeds of the tepary bean (*Phaseolus acutifolius*) and mulberry, *Morus* sp. (Rosales: Moraceae) seeds were purified by ConA Sepharose based affinity chromatography column. (Absar *et al.*, 2005; Richard *et al.*, 1990) However, in some contrast, there have been reports that affinity chromatography could not be applied successfully for the purification of some lectins. For instance, the isolation of *Dolichose biflorus* lectin with *N*-acetyl-galactosamine (NAG) immobilized to Sepharose was not successful and was caused by the substitution of the binding site at the C-6 hydroxyl of carbohydrate in the matrix. Rather, these lectins were resolved by affinity electrophoresis, a combination of affinity and conventional chromatography. (Borrebaeck and Etzler 1980) Another example is the lectin from ground elder (*Aegopodium podagraria*) rhizomes which also could not be purified by Gal-NAG-Sepharose, but by an affinity chromatography of erythrocyte membrane protein immobilized on cross-linked agarose. (Peumans *et al.*, 1985)
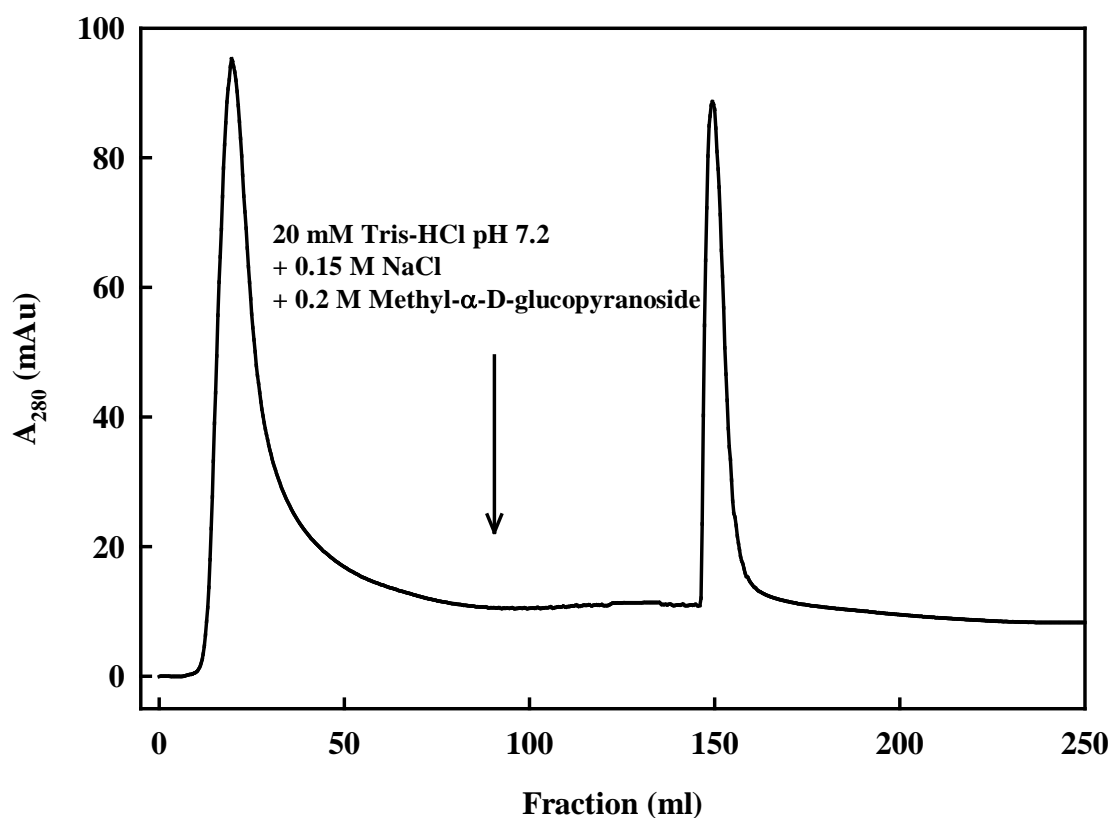
Figure 4.1. Affinity chromatogram *of A. jiringa* seed lectin on a ConA Sepharose column (1.6 × 20 cm) equilibrated and then washed (0-120 ml) with TBS. Lectin was then eluted with TBS containing 0.2 M methyl-α-D-glucopyranoside (120-250 ml) at a flow rate of 1.5 ml/min. Also shown is the hemagglutination activity for each eluted fraction. Data shown are representative of 3 such repeats.

Half Inhibition Concentration (IC_{50}) of *A. jiringa* protein is about 0.031±0.02 mg/ml. In addition the lectin from kidney beans (*Phaseolus vulgaris*) could inhibit the α-glucosidase enzyme too. This lectin from canned beans had inhibition for α-glucosidase is 70.6%. The raw kidney beans could inhibited the α-glucosidase enzyme about 77.1% (Shi *et al*., 2007). Moreover, protein extracts of *Sesbania grandiflora* flowers had reports that were α-glucosidase inhibitor proteins. The crude extract of proteins from 60% and 90% saturation precipitation had the inhibition for α-glucosidase were 49.55% and 82.07%, respectively (Boonmee *et al*., 2007).

4.2 Molecular weight determination

      The potential purity of the *A. jiringa* seeds α-glucosidase inhibitor proteins extract was evaluated at each step of the purification using SDS-PAGE under reducing conditions (Figure 4.2). The ammonium sulfate precipitated protein showed many protein bands of a medium molecular weight, with only a slight band at ~14-45 kDa. After the ConA-sepharose affinity purification step, a significant increase in the intensity of the 35.7 kDa band (estimated size) was seen, and only this band, suggesting a high degree of likely purity. The protein was found to contain 15.84% sugar. This protein had more sugar contain than Chinese evergreen chinkapin lectin (5.8%) (Wong *et al*., 2008) and *Arundu donex* lectin (2.1%) (Kaur *et al*., *2005*). It remains plausible that during the enrichment procedures prior to and during ConA Sepharose chromatography, residual endoglycanase activity, in conjunction with the preferential binding of the natural glycoprotein isoforms to the ConA resin, would select for purified glycoprotein of lower carbohydrate content than the real level. Conversely, we may have enriched for high carbohydrate content isoforms by the use of the ConA-Sepharose affinity chromatography.
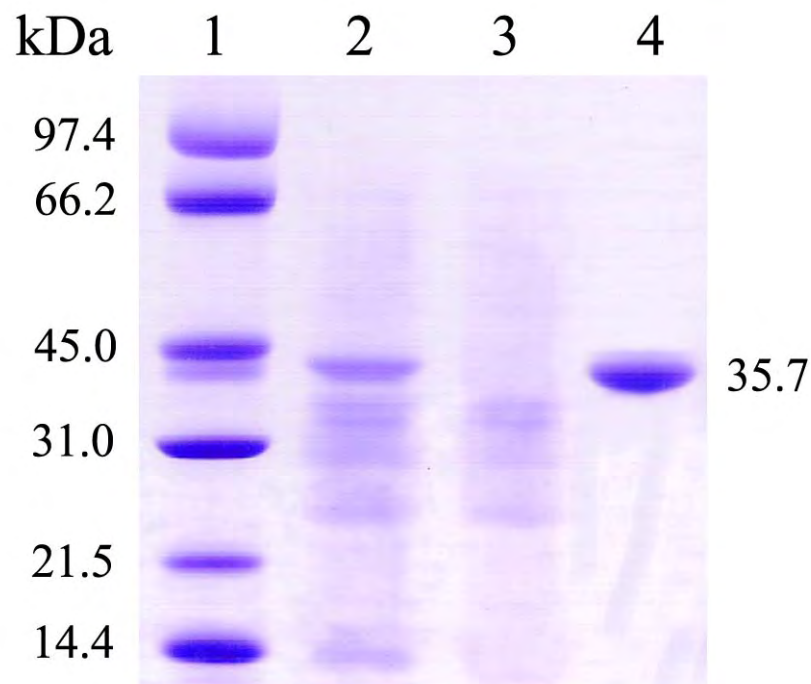
Figure 4.2 Reducing SDS-PAGE analysis of *A. jiringa* seed α-glucosidase inhibitor proteins purification where lanes 2-4 show 10☐μg of total protein. Lane 1, molecular weight standards; Lane 2, 90% saturation ammonium sulphate precipitated (kept) fraction; Lane 3, the non-bound ConA-sepahrose fraction; Lane 4, the eluted ConA sepahrose-bound fraction (enriched α-glucosidase inhibitor proteins preparation).

4.3 Protein concentration and α-glucosidase inhibitory activity

The α-glucosidase inhibitory activity of the α-glucosidase inhibitor has been tested at every step of the experiment. The bound fraction eluted from ConA sepharose has inhibitory activity against α-glucosidase at IC_{50} value of 0.031 ± 0.002 as shown in Table 4.1

Table 4.1 Summary of the enrichment of the α-glucosidase inhibitor

Purification step	Protein content (mg/ml)	IC_{50} (mg protein)
Crude extract	0.017 ± 0.002	0.25 ± 0.002
90% $(NH_4)_2SO_2$ precipitation	0.025 ± 0.004	0.19 ± 0.001
ConA Sepharose (bound fraction)	0.020 ± 0.003	0.031 ± 0.002

4.4 Effect of temperature on α-glucosidase inhibitory activity and thermo stability

No significant changes in the inhibition activity of the enriched α-glucosidase inhibitor protein fraction was seen when pretreated for 30 min within the temperature range of -20 – 80 °C, but from 90 °C upwards the observed α-glucosidase inhibitor activity decreased with increasing incubation temperature. (Figure 4.3A). In the case of a mannose/glucose-specific lectin from Chinese evergreen chinkapin (*Castanopsis chinensis*; CCL), Its activity was stable up to 60 °C for 30 min. Above this temperature, the activity underwent a decline (Wong *et al*., 2008). The thermal stability at various exposure times assay, the pretreatment of protein sample at 70 °C, 80 °C and 90 °C were chosen for investigation. From the experiment found that the pretreatment of protein sample at 70 °C, the inhibitor can be stable for 90 min. The pretreatment of protein sample at 80 °C, the inhibitor can be stable for 70 min. Whereas the pretreatment of protein sample at 90 °C, the inhibitor can be stable for only 20 min (Figure 4.3b). The thermal stability observed for this *A. jiringa* protein is comparable to that already reported for some other thermostable lectins treated under similar conditions. (Konozy *et al*., 2003; Oliveira *et al*., 2002). Indeed, the only thermophilic lectin isolated to date is from *Momordica charantia*, which has a maximal activity at 55 °C (Toyama *et al*., 2008).

Figure 4.3 (A) Effect of pretreatment temperature on the α-glucosidase inhibitor activity of the enriched α-glucosidase inhibitor protein fraction towards α-glucosidase. (B) Thermostability with increasing pretreatment time of the enriched α-glucosidase inhibitor protein fraction at: (●) 70 °C, (■) 80 °C and (▲) 90 °C on the subsequent α-glucosidase inhibitor α-glucosidase inhibitor activity against α-glucosidase. For both panels the data are shown as the mean ±1 SD and are derived from three repeats.

4.5 Effect of pH on α-glucosidase inhibitory activity

The α-glucosidase inhibitor proteins have high α-glucosidase inhibitory activity at pH 8-10 (Figure 4.4a). The α-glucosidase inhibitory activity was greatly high when pretreatment the protein sample with Tris-HCl pH 9. Whereas the pretreatment the protein sample with other buffer, glycine-HCl pH 2 - 4, sodium acetate pH 4 - 6, sodium phosphate pH 6 - 8, and glycine-NaOH pH 10 - 12 were none of activity. The pH stability at various exposure times assay, the pretreatment of protein sample with Tris-HCl buffer at pH 8 - 10 were chosen for investigation. From the experiment found that the inhibitor has high stability until pretreatment for 120 min. (Figure 4.4b).
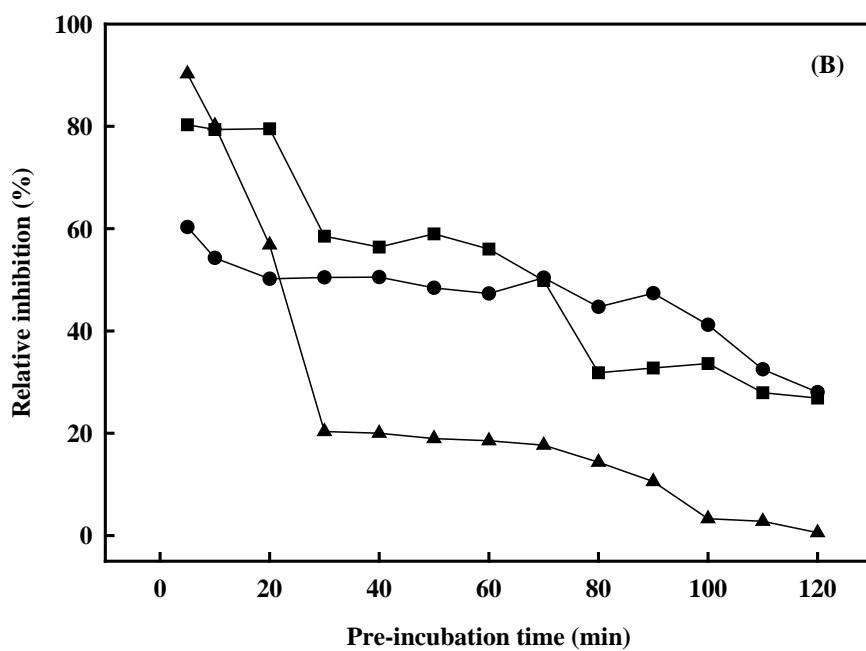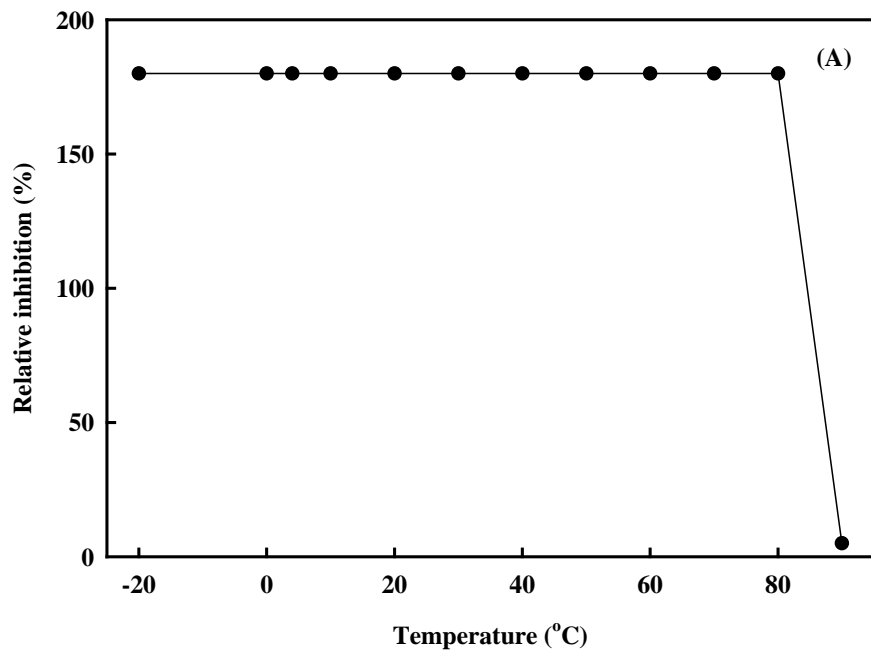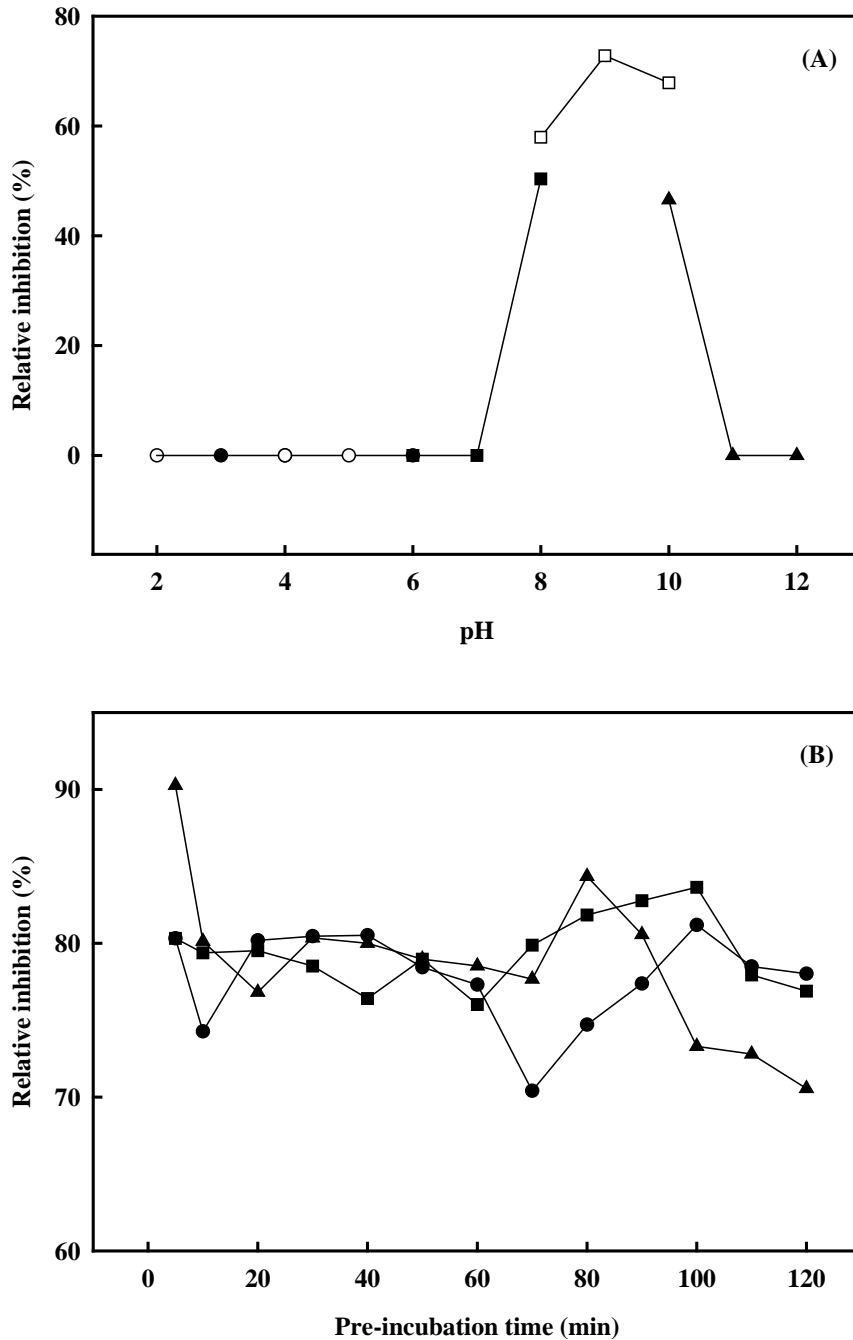
Figure 4.4 The effect of pH pretreatment on the α-glucosidase inhibitor activity of the enriched α-glucosidase inhibitor protein fraction against α-glucosidase. The data are shown as the mean ± 1 SD and are derived from three repeats. The following buffer systems were used: (●) 20 mM glycine-HCl (pH 2.0 - 4.0), (○) 20 mM sodium acetate (pH 4.0 - 6.0), (■) 20 mM potassium phosphate (pH 6.0 - 8.0), (□) 20 mM Tris-HCl (pH 8.0 - 1 0.0) and (▲) 20 mM glycine-NaOH (pH 10.0 - 12.0). (B) pH stability with increasing pretreatment time of the enriched α-glucosidase inhibitor protein fraction at pH: (●) 8.0, (■) 9.0, and (▲) 10.0 on the subsequent α-glucosidase inhibitor α-glucosidase inhibitor activity against α-glucosidase. For both panels the data are shown as the mean ±1 SD and are derived from three repeats.

4.6 Effect metal ion on α-glucosidase inhibitory activity

The effect of divalent cations on *A. jiringa* α-glucosidase inhibitor was evaluated with six different divalent metal ions. Cu^{2+} and Zn^{2+} were found to satisfy the requirements for alpha-glucosidase inhibitory activity, whereas Ca^{2+}, Mn^{2+} and Mg^{2+} were not able to support inhibitory activity and Fe^{2+} was able to support it at concentrations of above 50 mM (Table 4.1).

Table 4.2 The effect of divalent metal cations on the α-glucosidase inhibitory activity

Concentration (mM)	Metal salt					
	Mg^{2+}	Mn^{2+}	Fe^{2+}	Zn^{2+}	Ca^{2+}	Cu^{2+}
5	47.75	45	6.75	49.875	0	99
10	42.375	51.375	21.75	55.5	0	98.75
25	55.50	49.125	48.125	67.625	0	98.375
50	41.50	44.625	116.25	73.75	52.00	94.75
100	45.125	56.375	137.00	74.375	55.00	56.00

4.7 Mechanism of inhibition

Catalytic kinetic studies for α-glucosidase, with different substrate and enriched α-glucosidase inhibitor fraction concentrations were analyzed using Lineweaver-Burk equations (Figure 4.5). Both the maximal velocity (V_{max}, y-intercept) and the Michaelis-Menten constant (K_m, slope of the trend lines) decreased with increasing concentrations of the enriched α-glucosidase inhibitor protein fraction, and so this α-glucosidase inhibitor acted as a non-competitive inhibitor of the tested α-glucosidase. Non-competitive inhibitors do not compete with the substrate to bind to the active region of the free enzyme, but bind to enzyme-substrate complex, resulting in an enzyme-substrate inhibitor complex. For this reason, inhibition cannot be overcome by increasing the concentration of substrate. When the concentration of the α-glucosidase inhibitor was plotted against $1/V_{max}$ (observed), the K_i value was determined as 1.887 µg protein/ml via non-linear regression using the least squares difference method.

Inhibition of α-glucosidase by different compounds is described in the literature (Kim *et al*., 2005; Shim *et al*., 2003; Tadera *et al*., 2006). α-Glucosidase was effectively inhibited by naringenin, kaempferol, luteolin, apigenin, (+)-catechin/ (-)-epicatechin, diadzein and epigallocatechin gallate (Tadera *et al*., 2006). These flavonoids exhibited a mixed and close to non-competitive type of inhibition on yeast α-glucosidase. However, a combination of non-competitive and uncompetitive inhibition was observed in the study of α-glucosidase inhibition of pine bark extract against yeast *S. cerevisae* α-glucosidase (Kim *et al*., 2005). The non-competitive nature of the inhibition of α-glucosidase was reported for *Rhus chinensis* extract, a Korean herb traditionally used in the treatment of type 2 diabetes in Korea (Shim *et al*., 2003).
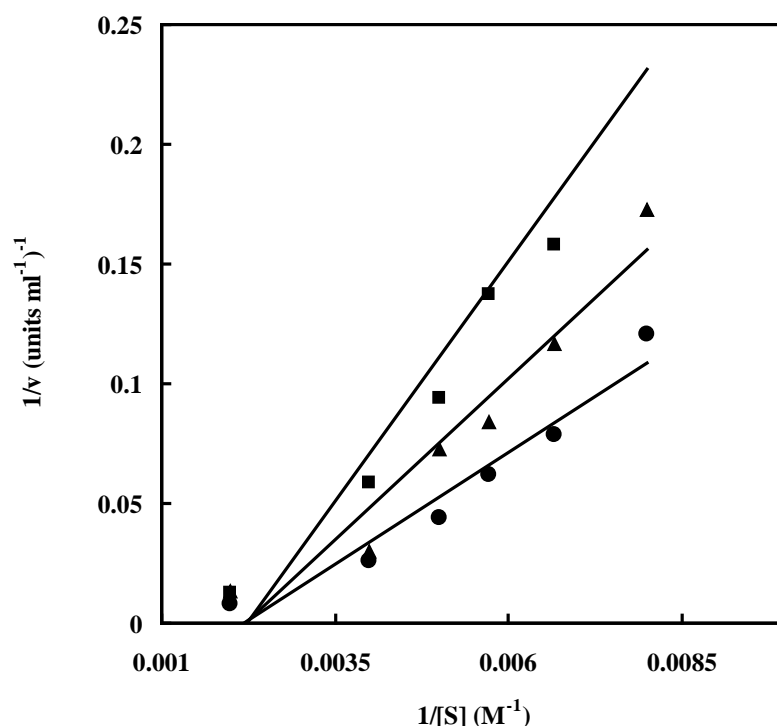
Figure 4.5 Lineweaver-Burk plots derived from the inhibition of α-glucosidase by the enriched α-glucosidase inhibitor protein fraction from *A. jiringa*. α-Glucosidase was treated with each indicated concentration of PNPG solution at one of 0.025 - 0.2 mM in the ◆) absence and presence of the enriched α-glucosidase inhibitor protein fraction at (■) 0.05 and (▲) 0.075 mg protein/ml. Data are shown as the mean ± 1 SD, derived from 3 repeats.

4.8 Potential α-glucosidase protein identification

The sequence analysis of a partial internal fragment of the purified protein from *A. jiringa* seeds obtained by in gel digestion with trypsin and subsequent sequence analysis with LC-MS/MS, revealed a peptide fragment with the likely sequence **VSSDG SPQGS SVGR** (Figure 4.6a). Comparisons to all protein sequences in the SwissProt database using BLASTP searching identified this fragment as a likely homolog of parts of a lectin precursor from the common bean, *Dioclea guianensis*. The high degree of internal amino acid sequence identity between the peptide fragment from *A. jiringa* protein, with those of other members of the lectin precursor family (Figure 4.6b) suggests that this protein could be a member of this lectin family as well.

		1				5					10					15				Accession Number
Archidendron jiringa lectin		R	V	S	S	D	G	S	P	Q	G	S	S	V	G	R	A			
Lectin precursor (*Dioclea guianensis*)	68	R	V	S	S	D	G	S	P	Q	G	S	S	V	G	R	A	83	A9J248
Concanavalin-A precursor (Con A) (*Canavalia gladiata*, Sword bean)	69	R	V	S	S	N	G	S	P	Q	G	S	S	V	G	R	A	84	P14894
Concanavalin-A precursor (Con A) (*Canavalia ensiformis*, Jack bean)	69	R	V	S	S	N	G	S	P	Q	G	S	S	V	G	R	A	84	P02866
Lectin alpha chain (*Dioclea guianensis*)	159	-	V	S	S	S	G	D	P	Q	G	S	S	V	G	R	A	173	P81637
Lectin alpha chain (*Dioclea rostrata*)	159	-	V	S	S	S	G	D	P	Q	G	N	S	V	G	R	A	173	P58908
Lectin alpha chain (*Cratylia floribunda*)	158	R	V	S	-	N	G	S	P	Q	S	N	S	V	G	R	A	172	P81517
Mannose/glucose-specific lectin (*Cratylia mollis*, Camaratu bean)	156	R	V	S	-	N	G	S	P	Q	S	N	S	V	G	R	A	170	P83721
Mannose-specific lectin (*Chimonanthus praecox*)	101	L	Y	S	S	Q	G	S	A	I	W	S	S	K	T	W	Q	117	A2SVT1
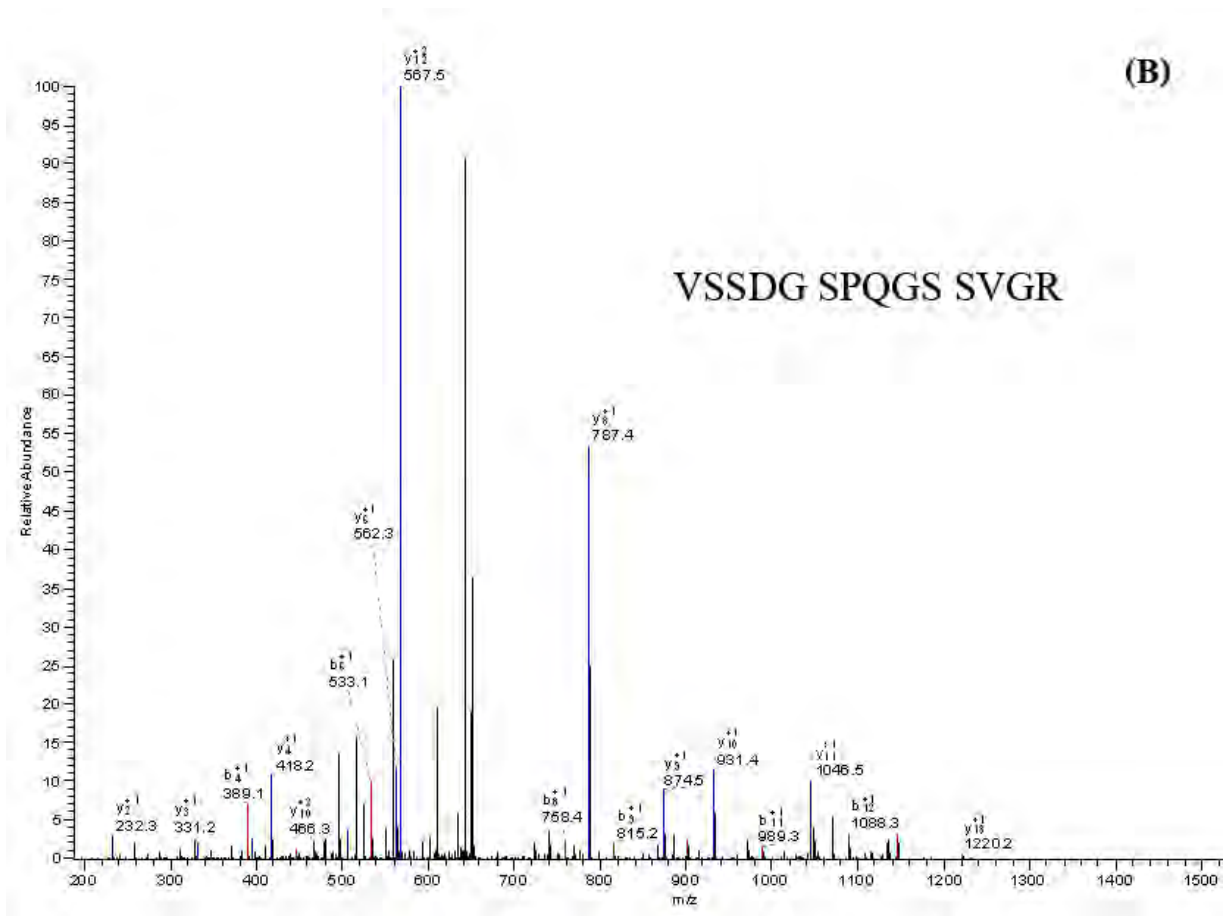
Figure 4.6 (A) Amino acid sequences from the tryptic fragments of the purified *A. jiringa* seed α-glucosidase inhibitor protein. Comparisons are made with other lectins from the mannose-glucose specific lectin family that showed the highest sequence homology in BLASTP searches of the NCBI and SwissProt databases. Shaded regions represent regions of identity. (B) LC/MS/MS spectra of the tryptic digest of the purified α-glucosidase inhibitor protein used to derive the data in (A) above.

4.9 Biospecific interaction determination by surface plasmon resonance (SPR)

Biospecific interactions, between α-glucosidase inhibitor from seed of *A. jiringa* and α-glucosidase, studies were performed on Autolab ESPRIT system. Biosensor system was based on the principle of surface plasmon resonance (SPR). The various concentrations of inhibitors in 20 mM phosphate buffer, pH 7.2 were coupled to a certified grade 11 MUA gold plate. The unreacted groups, on the surface of the gold, were blocked with ethanolamine. A blank channel was using phosphate buffer as a control. All measurements were analyzed by 1U/ml α-glucosidase enzyme.

The analysis of interaction between protein sample, concentration arrange from 0.001 mg to 0.028 mg, and α-glucosidase enzyme shown in Table 4.3. Result that the protein concentration of 0.017 mg has highest interaction with α-glucosidase enzyme as shown in Table 4.3 and Figure 4.7. From the experiment found that the α-glucosidase inhibitor has specific interaction with α-glucosidase with affinity constant $= 9.3773 \times 10^{-7}$ s^{-1}, $K_s = 0.0241$ s^{-1}, $K_a = 2.39 \times 10^{3}$ $s^{-1}M^{-1}$, and $K_d = 0.0117$ M. The presented analytical system based on a surface plasmon resonance system and is a valuable tool for the characterization of α-glucosidase inhibitor especially their binding-domains. This can be done by analyzing the initial binding rate and calculating the K_d value.

Table 4.3 Analysis of interaction between α-glucosidase and its inhibitor

Protein concentration (mg)	Response (m°)
0.028	146.8
0.022	175.9
0.017	183.4
0.011	174.9
0.006	123.4
0.003	110.5
0.001	107.9
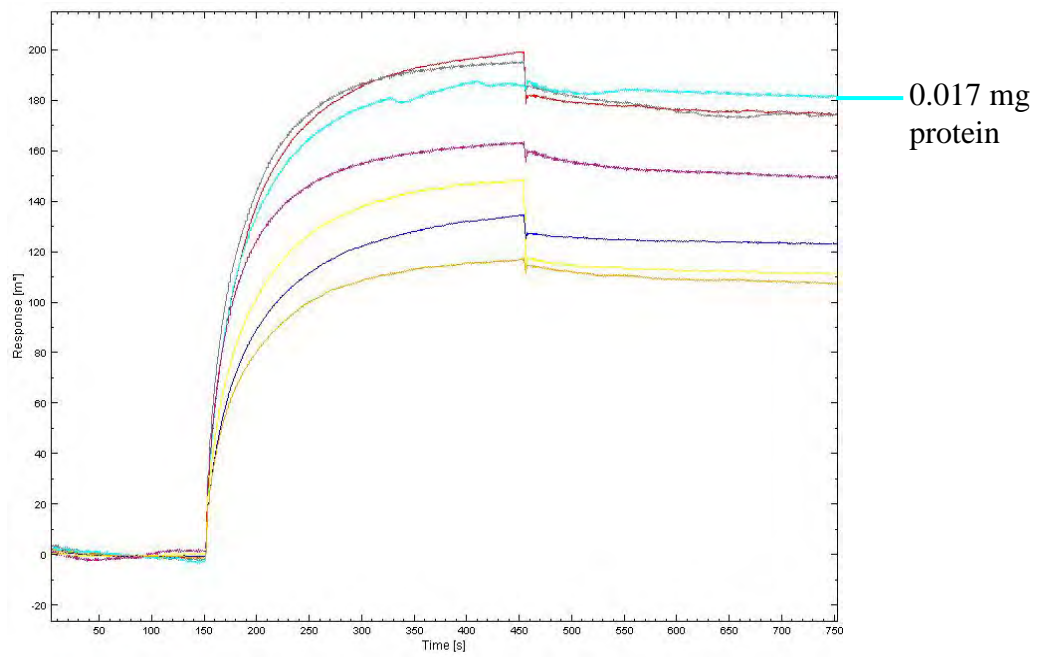
Figure 4.7 The representative sensorgrams interactions of protein sample concentration from 0.001 – 0.028 mg and analyzed with 1U/ml α-glucosidase enzyme.

CHAPTER V

CONCLUSION

In this study, the protein from seeds of *A. jiringa* was purified by affinity chromatography using ConA Sepharose to apparent homogeneity. The purified protein has α-glucosidase inhibitory activity at IC_{50} value of 0.031 ± 0.02 mg protein. The molecular mass of the purified protein was 35.7 kDa, as estimated by SDS-PAGE. The protein was thermostable up to 80 ^{o}C for 70 min and showed an optimum activity within the pH range of 8.0-10.0 with Tris-HCl buffer and it has stability up to 120 min. Divalent cations appear to be essential for the activity of this protein which is Cu^{2+}, Fe^{2+} and Zn^{2+}. Fe^{2+} gave high activity when used in high concentration; more than 50 mM. The internal amino acid sequence of the protein showed similarity to the sequences of the lectin precursor from *Dioclea guainensis*. Furthermore, this α-glucosidase inhibitor has high specific interaction with α-glucosidase enzyme. This test was performed by surface plasmon resonance (SPR) technique with affinity constant $= 9.3773 \times 10^{-7}$ s^{-1}, $K_s = 0.0241$ s^{-1}, $K_a = 2.39 \times 10^{3}$ $s^{-1}M^{-1}$, and $K_d = 0.0117$ M.

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APPENDICES

APPENDIX A

Preparation for denaturing polyacrylamide gel electrophoresis

1. Stock solutions

2 M Tris-HCl (pH 8.8)

    Tris (hydroxymethyl)-aminomethane   24.2 g

    Adjusted pH to 8.8 with 1 M HCl and adjusted volume to 100 ml with distilled water

1 M Tris-HCl (pH 6.8)

    Tris (hydroxymethyl)-aminomethane   12.1 g

Adjusted pH to 6.8 with 1 M HCl and adjusted volume to 100 ml with distilled water.

10% SDS (w/v)

    Sodium dodecyl sulfate (SDS)   10 g

50% Glycerol (w/v)

    100% Glycerol   50 ml

    Added 50 ml of distilled water

1% Bromophenol blue (w/v)

    Bromophenol blue   100 mg

    Brought to 10 ml with distilled water and stirred until dissolved.

    Filtration will remove aggregated dye.

2. Working solution

Solution A (30% (w/v) acrylamide, 0.8% (w/v) bis-acrylamide)
Acrylamide	29.2 g
N,N,-methylene-bis-acrylamide	0.8 g
Adjust volume to 100 ml with distilled water	

Solution B (1.5 M Tris-HCl pH 8.8, 0.4% SDS)
2 M Tris-HCl (pH 8.8)	75 ml
10% SDS	4 ml
Distilled water	21 ml

Solution C (0.5 M Tris-HCl pH 6.8, 0.4% SDS)
1 M Tris-HCl (pH 6.8)	50 ml
10% SDS	4 ml
Distilled water	46 ml

10% Ammonium persulfate
Ammonium persulfate	0.5 g
Distilled water	5 ml

Electrophoresis buffer (25 mM Tris, 192 mM glycine, 0.1% SDS)
Tris (hydroxymethyl)-aminomethane	3 g
Glycine	14.4 g
SDS	1 g
Dissolved in distilled water to 1 litre without pH adjustment	
(final pH should be 8.3)	

5x sample buffer
(60 mM Tris-HCl pH 6.8, 25% glycerol, 2% SDS, 0.1% bromophenol blue, 14.4 mM 2-mercaptoethanol)
1 M Tris-HCl (pH 6.8)	0.6 ml
Glycerol	5 ml

10% SDS	2	ml
1% Bromophenol blue	1	ml
2-mercaptoethanol	0.5	ml
Distilled water	0.9	ml

3. SDS-PAGE

15% Separating gel

Solution A	10.0	ml
Solution B	5.0	ml
Distilled water	5.0	ml
10% Ammonium persulfate	100	μl
TEMED	10	μl

5.0% Stacking gel

Solution A	0.67	ml
Solution B	1.0	ml
Distilled water	2.3	ml
10% Ammonium persulfate	30	μl
TEMED	5.0	μl

APPENDIX B

Calibration curve for protein determination by Bradford method

Y = 0.0155X
R² = 0.9881

A₅₉₅ (nm)

BSA (µg)
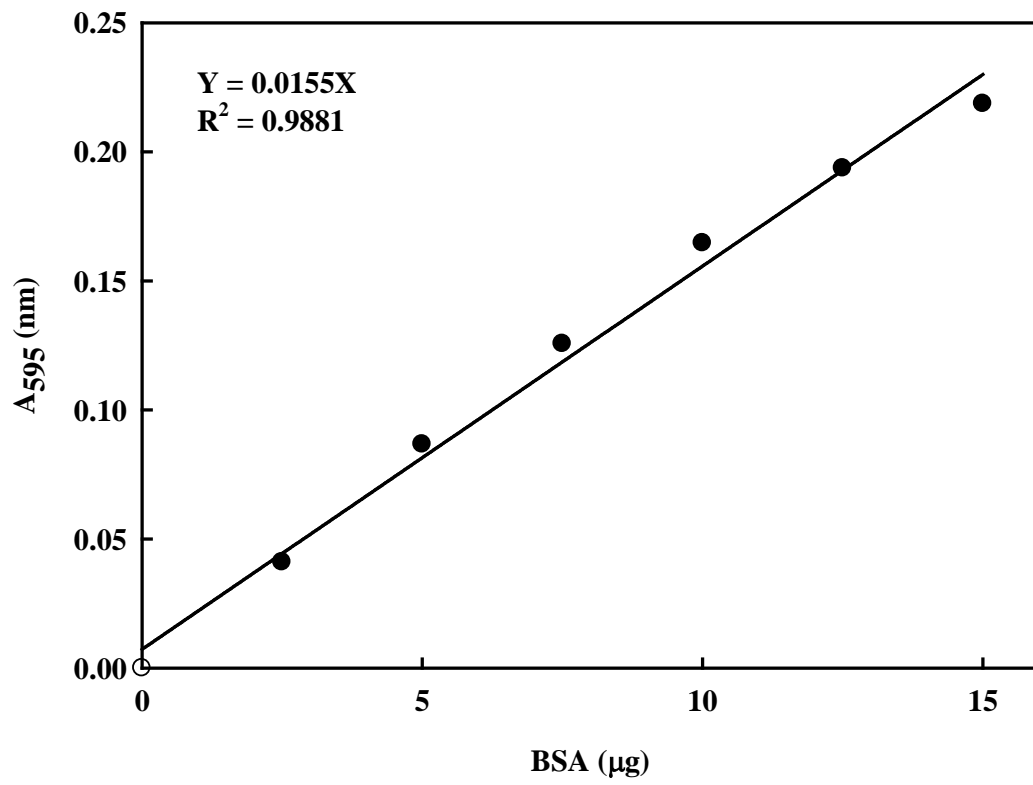
APPENDIX C

Calibration curve for carbohydrate content by Dubois method

$$Y = 0.003X$$
$$R^2 = 0.9916$$

(Plot: A_{490} (nm) on the y-axis from 0.00 to 0.20 versus Glucose (µg) on the x-axis from 0 to 50.)
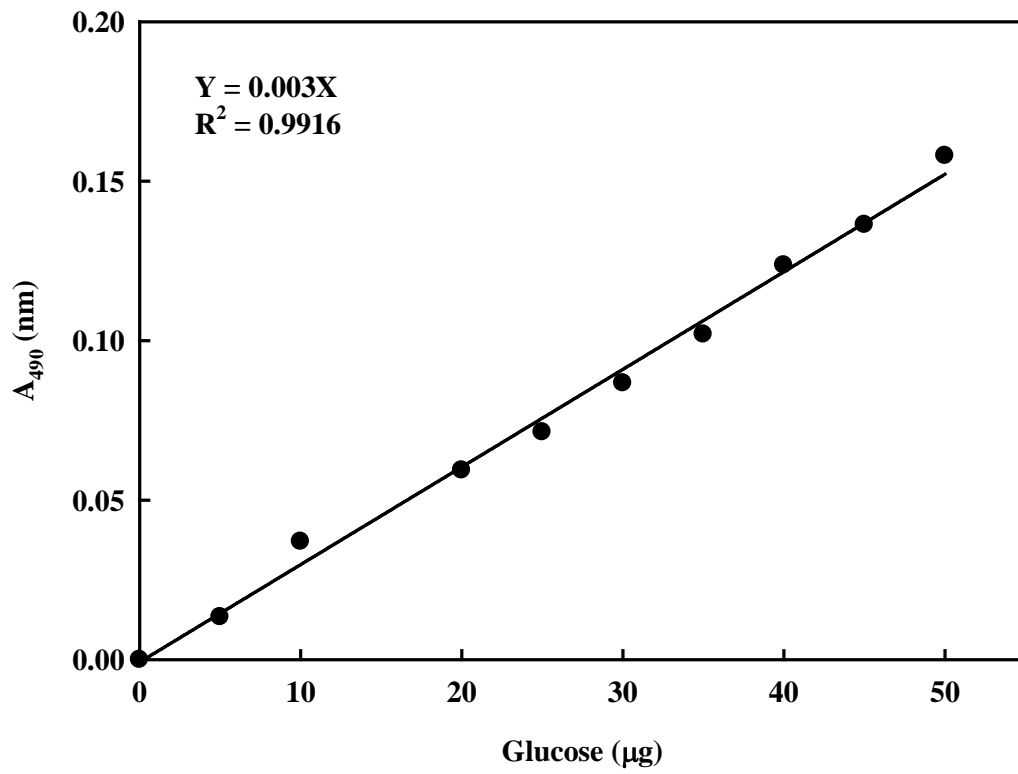
APPENDIX D

Amino acid abbreviations

Amino acid	Three-letter	One-letter
Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartic-acid	Asp	D
(Asn + Asp)	Asx	B
Cysteine	Cys	C
Glutamine	Gln	Q
Glutamic acid	Glu	E
(Gln + Glu)	Glx	Z
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V

BIOGRAPHY

Miss Panadda Virounbounyapat was born on November 28, 1978 in Pattani, Thailand. She graduated with Bachelor Degree of Science (Medical Technology) from Faculty of Medical Technology, Chiang Mai University in 2000. She works at Department of Medical Sciences, Ministry of Public Health from 2000 to present. She was admitted to the Master degree of Science in Biotechnology, Faculty of Science, Chulalongkorn University in 2008.

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