การทำให้บริสุทธิ์และลักษณะสมบัติของเลคตินจากเหง้าของกระชายดำ

Kaempferia parviflora. Wall. Ex. Baker

นางสาววิชชุลดา ก่อนกำเนิด

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
สาขาวิชาเทคโนโลยีชีวภาพ
คณะวิทยาศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2552

PURIFICATION AND CHARECTERIZATION LECTIN FROM THE
RHIZOME OF *Kaempferia parviflora* Wall. Ex. Baker

Miss Wichchulada Konkumnerd

A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science Program in Biotechnology
Faculty of Science Chulalongkorn University
Academic Year 2009

Thesis Title	PURIFICATION AND CHARACTERIZATION OF LECTIN
	FROM RIZOMES OF *Kaempferia parviflora* Wall. Ex. Baker
By	Miss Wichchulada Konkumnerd
Field of Study	Biotechnology
Thesis Advisor	Associate Professor Polkit Sangvanich, Ph.D.
Thesis Co-Advisor	Aphichart Karnchanatat, Ph.D.

Accepted by the Faculty of Science, Chulalongkorn University in Partial Fulfillment of the Requirements for the Master's Degree
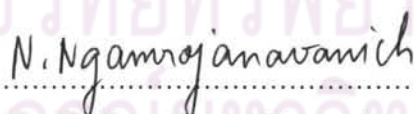
.... Dean of the Faculty of Science

(Professor Supot Hannongbua, Dr. rer. nat.)

THESIS COMMITTEE

.... Chairman

(Associate Professor Amorn Petsom, Ph.D.)

.... Thesis Advisor

(Associate Professor Polkit Sangvanich, Ph.D.)

.... Thesis Co-Advisor

(Aphichart Karnchanatat, Ph.D.)

.... Examiner

(Associate Professor Nattaya Ngamrojanavanich, Ph.D.)

.... External Examiner

(Chantragan Phiphobmongkol, Ph.D.)

วิชชุลดา ก่อนกำเนิด: การทำให้บริสุทธิ์และลักษณะสมบัติของเลคตินจากเหง้าของกระชายดำ *Kaempferia parviflora* Wall Ex. Baker (PURIFICATION AND CHARACTERIZATION OF LECTIN FROM THE RHIZOMES OF *Kaempferia parviflora* Wall. Ex. Baker) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: รศ.ดร. พลกฤษณ์ แสงวณิช, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: ดร. อภิชาติ กาญจนทัต, 73 หน้า.

ในงานวิจัยนี้ได้ทำการศึกษาลักษณะสมบัติของเลคตินจากเหง้าของกระชายดำ *Kaempferia parviflora* Wall Ex. Baker โดยการนำเหง้าของกระชายดำมาสกัดด้วยสารละลายทริสบัฟเฟอร์ที่ค่าความเป็นกรดด่าง 7.2 จากนั้นนำโปรตีนมาทำให้บริสุทธิ์โดยการตกตะกอนด้วยเกลือแอมโมเนียมซัลเฟตอิ่มตัวที่ 80 เปอร์เซนต์ ทำเลคตินให้บริสุทธิ์โดยเทคนิคโครมาโทกราฟีแบบสัมพรรคภาพด้วยคอลัมน์ ConA Sepharose และ โครมาโคกราฟีแบบเจลฟิลเตรชันด้วยคอลัมภ์ Sephacryl S100 ตามลำดับ เมื่อใช้เทคนิคพอลิอะคริลาไมด์เจลอิเล็กโตรฟอเรซิสแบบเสียสภาพ ซึ่งเลคตินบริสุทธิ์ที่ได้มีน้ำหนักโมเลกุลประมาณ 41.7 กิโลดาลตัน และมีกิจกรรมการเกาะกลุ่มของเซลล์เม็ดเลือดแดงของกระต่ายมากที่สุด รองลงมาได้แก่ เซลล์เม็ดเลือดแดงของแกะ ห่าน หนูแรท หนูเมาส์ และหนูตะเภาตามลำดับ รวมถึงมีกิจกรรมการเกาะกลุ่มของเซลล์เม็ดเลือดแดงของคนในระบบเลือดเอบีโอโดยมีปฏิกิริยากับเม็ดเลือดแดงของหมู่เลือดโอมากที่สุด รองลงมาได้แก่ หมู่เลือดเอ บี และ เอบี ซึ่งมีกิจกรรมเท่ากัน เลคตินมีความเสถียรต่อค่าความเป็นกรด-ด่างในช่วง 6 จนถึง 8 และมีความเสถียรต่อความร้อนที่อุณหภูมิ 75 องศาเซลเซียส นอกจากนี้เลคตินสามารถยับยั้งการเจริญเติบโตของราโรคพืช ได้แก่ *Exserohilum turcicum*, *Fusarium oxysporum* และ *Colectrotrichum cassicola* ที่ความเข้มข้น 18 จนถึง 36 ไมโครกรัม รวมทั้งสามารถยับยั้งการเจริญเติบโตของแบคทีเรีย ทั้งแบคทีเรียแกรมลบ (*Pseudomonas aeruginosa*) และแกรมบวก (*Bacillus subtilis* และ *Staphylococcus aureus*) อย่างไม่จำเพาะที่ความเข้มข้น 1.270, 1.270 และ 0.184 ไมโครกรัมตามลำดับ และค่าความเข้มข้นการยับยั้งการทำงานของแอลฟา- กลูโคซิเดสที่ 50 เปอร์เซนต์ เท่ากับ 0.04 มิลลิกรัม

สาขาวิชา: เทคโนโลยีชีวภาพ	ลายมือชื่อนิสิต	วิชชุลดา ก่อนกำเนิด
ปีการศึกษา: 2552	ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก	
	ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์ร่วม	

5072462523   : MAJOR BIOTECHNOLOGY

KEYWORDS : *Kaempferia parviflora* / Lectin / Biological activity / Purification

WICHCHULADA KONKUMNERD : PURIFICATION AND CHARECTERIZATION LECTIN FROM THE RHIZOME OF *Kaempferia parviflora* Wall. Ex. Baker THESIS ADVISOR : ASSOC. PROF. POLKIT SANGVANICH, Ph.D., THESIS CO-ADVISOR : APHICHART KARNCHANATAT, Ph.D., 73 pp.

A lectin was isolated from the rhizomes of black galingale (Kra Chai Dam), *Kaempferia parviflora* Wall. Ex. Baker, using, in sequential order, ammonium sulfate precipitation, Concanavalin A affinity chromatography and Sephacryl S-100 gel filtration chromatography. The molecular weight of the purified lectin (or monomeric subunit) was estimated to be about 41.7 kDa by reducing SDS-PAGE analysis. The lectin showed hemagglutinating activity against, in order of the strongest activity, rabbit >> sheep > goose = rat > mouse = guinea pig = human O group > human A = B = AB group erythrocytes. In terms of hemagglutination activity, the optimal pH range of the *K. parviflora* lectin was between pH 6 - 8 and it was active up to 75 °C. Furthermore, the purified *K. parviflora* rhizome lectin preparation at 18 - 36 μg /0.3 cm^2 disc was able to inhibit the growth of the plant pathogenic fungi, *Exserohilum turcicum, Fusarium oxysporum* and *Colectrotrichum cassicola*, on potato dextrose agar (PDA) plates, and showed potentially non-selective antibacterial activity since it inhibited the growth of both gram positive (*Bacillus subtilis* and *Staphylococcus aureus*) and gram negative (*Pseudomonas aeruginosa*) bacteria with a minimum inhibitory concentration (MIC) of 0.184, 1.270 and 1.270 mg/ml, respectively. Finally, this purified lectin also exhibited a strong α-glucosidase inhibitory activity, but with a relatively high IC$_{50}$ value of 0.04 μg/ml.

Field of study: _____Biotechnology_____ Student's signature: _____วิชชุลดา กอนคำเหลอ_____

Academic year: _____2009_____ Advisor's signature: _____Polkit Sangvanich_____

Co-advisor's signature: _____Aphichart Karnchanatat_____

ACKNOWLEDGEMENTS

I am especially grateful to my advisor and co-advirsor, Associate Professor Dr. Polkit Sangvanich and Dr. Aphichart Karnchanatat for their valuable guidance and assistance throughout my studies and research at Chulalongkorn University. I wish to thank my graduate committee member, Associate Professor Dr. Amorn Petsom, Associate Professor Dr. Nattaya Ngamrojnavanich, Dr. Chantrakan Thiphopmongkol for their helpful advice and comments.

I would to thank the Faculty of Science, Graduate School, Chulalongkorn University, Programe of Biotechnology, Faculty of Science for financial support, I am also thanking the the Institute of Biotechnology and Genetic Engineering, Chulalongkorn University for laboratory facilities. I thank all of my friends, all member in Program of Biotechnology and in the Institute of Biotechnology and Genetic Engineering for their friendship and helps during my graduate studies.

Additionally, I would like to thank Miss. Apaporn Boonmee in Protein Unit Reseach centre for Bioorganic Chemistry for her teaching and good performance, Mrs. Supissara Techaprempreecha for her helping and bacterial stock for antibacterial experimental.

Finally, I would like to appreciate my family for their great support and encouragement throughout my education.

CONTENTS

LIST OF TABLES

LIST OF FIGURE

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
HU	Hemagglutinating units
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
PDA	Potato dextrose agar
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

ศูนย์วิทยทรัพยากร
จุฬาลงกรณ์มหาวิทยาลัย

CHAPTER I

INTRODUCTION

Lectins are polyvalent sugar-binding proteins or glycoproteins of non-immune origin that have an apparent ubiquitous distribution in nature, a specificity for terminal or subterminal carbohydrate residues and can agglutinate cells and/or precipitate glycoconjugates (Goldstein *et al*., 1980). The main characteristic of this class of proteins is their ability to interact with carbohydrates and thus combine with the glycocomponents of the cell surface, as well as with cytoplasmic and nuclear structures and the extracellular matrix of cells and tissues from throughout the five kingdoms (Brooks and Leathem, 1998). The availability of a large number of lectins with distinct carbohydrate specificities has resulted in the use of these proteins as tools in medical and biological research (Singh *et al*., 1999), and has attracted considerable interest because of their remarkable effects in a wide range of biological systems, including the purification and characterization of glycoconjugates and the study of cell-surface architecture (Lis and Sharon, 1986).

Although ubiquitously distributed, plants remain the most frequently used source of lectin studies due to both the ease of their extraction and the relatively high yields that can be obtained. Moreover, different families of plants, as well as different tissues within the same plant, can contain different lectins and bioactivities, including different carbohydrate-binding specificities. It has been suggested that plant lectins may have important roles according to their abundance, including in the plant immune defence, and also that lectins have been co-opted adapted for several functions during evolution (Etzler, 1985). Moreover, plant lectins have become of increasing interest in recent years, mainly due to the discovery of their potent biological activities (Wood *et al*., 1999), leading to different lectins being purified and characterized in some detail with respect to their biochemical properties and carbohydrate binding specificity (Goldstein and Potetz, 1986; Peumans and van Damme, 1993). Although plant lectins represent a large group of plant proteins, to date they have been found in less than 500 species, which may indicate that only a limited number of higher plants contain detectable levels of lectins (Van Damme *et al*., 1998), or simply reflect a lack of systematic studies of plants. Within such a potential sampling bias, the majority of the

studies on lectins have been carried out on species within the Fabaceae (= Leguminosae) family, the so called legume species (Kocourek, 1986; Lakhtin, 1994), and particularly within legume seeds where they comprise up to 15% (w/w) of the total seed protein. However, as a result of these albeit selective studies, plant lectins have become a very popular class of proteins because of their obvious potential as tools, as described above, to aid researchers in other areas of the life sciences. With respect to plant immunity, a variety of lectins are presently envisioned to be involved in one or more of at least three plant defense roles. The first such defense role envisaged for some lectins is in the recognition of oligosaccharide signals produced by the breakdown of cell wall components of the plant or pathogen upon contact with the plant. The second type of defense role involves the direct interaction of the lectin with the infectious agent. Finally, the third defense type, with considerable support, is that some lectins play a direct role in protecting the plant from animal predators (Weis and Drickamer, 1996).

The family Zingiberaceae is a large, important and well known monocot family (ginger plants) that is conspicuous throughout the tropics. It is comprised of approximately 52 genera and 1,400 species, with the center of diversity from South to Southeast Asia. In Thailand, there are 26 genera of more than 300 species of these plants (Larsen and Larsen, 2006), and many of these are used in traditional medicine (Sirirugsa, 1998). The black galingale, *Kaempferia parviflora* Wall ex. Baker, locally known in Thai as Kra-Chai-Dam, is an herbaceous plant within the Zingiberaceae family, and its black to purple rhizomes have been traditionally employed in folk medicine as a medicinal plant for treatment of a wide spectrum of illness. Since ancient times, it has traditionally been used as a health promoting and vitalizing agent (Sennil and Trichalee, 2003; Yenjai *et al*., 2004). For example, they or their ethanolic extracts, are applied as a general health promoting agent, as an anti-inflammatory agent and for the treatment of gastrointestinal disorders (Rujjanawate *et al*., 2005; Yenjai *et al*., 2004). Their purported therapeutic activities have attracted a great deal of interest in recent years. A preliminary study on *K. parviflora* plantlets revealed the presence of hemagglutinating activity against rabbit erythrocytes in rhizome extracts (Sangvanich *et al*., 2007). Consequently, the aims of this study were purified, characterized a lectin from the rhizomes of *K. parviflora* rhizomes as a prerequisite to further study this lectin with respect to its physiological role in the plant.

CHAPTER II

LITERATURE REVIEW

2.1 Zingiberaceae

The Zingiberaceous plants are characterized by their tuberous or non-tuberous rhizomes, which have strong aromatic and medicinal properties. It is commonly knownas ginger, and exists in about 50 genera and 1,300 species worldwide, distributed mainly in South and Southeast Asia. Turmeric of Zingiberaceous plants in powder form is widely applied as a food additive in many Asian countries. Medicinal functions for treatment of diseases such as diarrhea, coryza, dermatosis disorders and rheumatism are also widely mentioned in traditional remedies. Zingiberaceae plants contain many essential oils, including terpenes, alcohols, ketones, flavonoids, carotenoids and phytoestrogens. For instance, the water extract of *Zingiber officinale* exhibits 6-Gingerol, and is mostly found in the rhizome in concentrations of 130-7,138 ppm. The major functional compound in Curcuma turmeric is curcumin, which has concentrations as high as 38,000 ppm in certain species. Less-polar constituents including curcuminoids, kava pyrones and gingerols isolated from Zingiberaceous plants, which have been reported for their biological activities in antifungal, antioxidant, insecticidal, and anti-inflammatory activities are particularly important and relevant for these applications (Chen *et al*., 2008). Chloroformic extracts of selected Thai medicinal plants commonly employed to treat infections were investigated for their antibacterial activity against important foodborne pathogenic bacteria. These included *Bacillus cereus, Staphylococcus aureus,* methicillin-resistant *S. aureus* (MRSA), *Escherichia coli* O157:H7*, Salmonella typhi* and *Shigella* sp. Among 33 extracts tested, only chloroformic extracts of five plant species exhibited antibacterial properties (Voravuthikunchai *et al*., 2006).

Twelve compounds were isolated from *Alpinia mutica* Roxb., *Kaempferia rotunda* Linn., *Curcuma xanthorhiza* Roxb., *Curcuma aromatica* Valeton and *Zingiber zerumbet* Smith (Family: Zingiberaceae) and three synthesized derivatives of xanthorrhizol were evaluated for their ability to inhibit arachidonic acid- (AA), collagen- and ADP-induced platelet aggregation in human whole blood (Jantan *et al.* 2008). Crude organic of *Curcuma longa* L. extracts of turmeric were capable of

inhibiting LPS-induced TNF-a, PGE2 production and can inhibited COX-2 expression (Jantan *et al*., 2008; Lantz *et al*., 2005). Zingiberaceae plants from Taiwan area were collected and analyzed for their functional properties. Antioxidant performances were best observed in *Vanoverberghia* and *Hedychium*, both 89%, and DPPH scavenging activity followed similar trends. Particularly, *Zingiber oligophyllum*, considered as a traditional medicinal plant used in Taiwan exhibited low DPPH scavenging activity and reducing power (Chen *et al*., 2008). Crude extracts of four spices in the Zingiberaceae (galangal, ginger, turmeric and krachai) were evaluated in their ability to inhibit the growth of test microorganisms and to assess cell morphological changes. The ginger water-extracted at a concentration of 5.00 mg/ml was found to have strong inhibitory effects on the growth of representative foodborne pathogens, *S. aureus* and *E. coli* (Oonmetta-aree 2005). A water extract of Curcuma longa L. (Zingiberaceae) (CLE), having O_2^- scavenging activity rescued PC12 cells from pyrogallol-induced cell death. Hypoxia/reoxygenation injury of PC12 cells was also blocked by Curcuma longa L. (Koo *et al*., 2004). The antibacterial activity of ginger, mangoginger and turmeric and mixtures thereof i.e.ginger and mangoginger, ginger and turmeric, turmeric and mangoginger were investigated. The 1, 4-Dioxan and 1, 4-Dioxan, *N,N*, dimethylformamide extracts of mangoginger showed the highest activity against *B. subtilis*. The antibacterial activity of ginger in 1, 4-Dioxan showed the highest activity against *S. aureus* and Extracts in *N,N,* dimethylformamide did not show any activity (Chandarana *et al*., 2005). Nitric oxide in the protective effect of Curcumin (*Curcuma longa*, Zingiberaceae) against 72-h sleep deprivation-induced behavioral alterations and oxidative damage in mice. Curcumin extract treatment significantly restored depleted reduced glutathione, catalase activity, attenuated raised lipid peroxidation and nitrite level as compared to control (72-h sleep-deprived) animals (Kumar *et al*., 2008).

2.2 *Kaempferia parviflora*

Kaempferia parviflora is one of the plants in the Zingiberaceae family, locally known in Thai as kra-chai-dam. The rhizomesas food ingredients and in Thai traditional medicine for treatment of allergy (Tewtrakul *et al*. 2008), gastrointestinal disorders (Vichitphan *et al*., 2007), fungal infection and impotence . This plant has been known as Thai ginseng. *K. parviflora* has recently been reported to possess antifungal (Wang *et al*., 2005) antimycobacterial, antiplasmodial (Yenjai *et al*., 2004),

anti-peptic ulcer (Rujjanawate *et al*. 2005) and anti-viral protease effects (Sookkongwaree *et al*., 2006) as well as modulators of multidrug resistance in cancer cells (Patanasethanont *et al*. 2007). *K. parviflora*, compound 5 (5- hydroxy-3,7,30,40-tetramethoxyflavone) exhibited the highest activity against the nitric oxide inhibitory effect, with an IC_{50} value of 16.1 lM (Tewtrakul *et al*., 2008). The active constituents for anti-allergic activity of *K. parviflora*, 5-hydroxy-3,7,3,4-tetramethoxyflavone, 5-hydroxy-7-methoxyflavone and 5-hydroxy-7,4-dimethoxyflavone are responsible for anti-allergic effect of this plant. And it long been used among Thai men for sexual enhancement , *K. parviflora* extract at 240 mg/kg BW reduced the time in the first 10 mintues of rat courtship behavior and the use of high and chronic doses of *K. parviflora* in humans should be considered inadvisable. Five flavonoid has been isolated from *K. parviflora* that exhibited antifungal activity against Candida albicans with IC50 value of 19.98 µg/ml, antimicrobacteria with a MIC value 50 µg/ml and showed antiplasmodial activity and cytotoxicity against KB, BC and NCl-Hl 87 cell line (Yenjai *et al*., 2007).

Figure 2.1 The fresh rhizome of *K. parviflora.*

2.3 Lectin

Lectins are glycoproteins that specific binding with carbohydrates and showed distinct structure with a high degree of specificity. It is usually found in plant, animal and microbial. The first of the animal lectins shown to be specific for a sugar (L-fucose) was from the eel. In another animal such as shrimps and claps (Crustacean), in microbial such as *E. coli* and *S. typhi* and plant such as leguminosae family. In 1952, of the first time that isolated lectin in mammalian is galactose-specific hepatic asialoglycoprotein receptor (Watkins *et al*., 1952). Although plant lectins are mostly isolated from seeds, also occurred in other plants tissues like leaves, roots, stem and tubers has been reported. Almost lectins consist of a small number of subunits, not essentially identical, of molecular weight usually below 40 kDa.

At present, the role of plant lectin is not distinct due to lectin capacity to discriminate of red blood erythrocyte. The concanavalin A is the first hemagglutinin that could purify from jack bean (*Canavalia ensiformis*) and it agglutinates cells such as erythrocytes and yeasts and also precipitates glycogen from solution. But it activity was inhibited by sucrose, demonstrating for the first time the sugar specificity of lectins. These result suggested that the hemagglutination induced in concanavalin A might be a consequence of a reaction of the plant protein with carbohydrates on the surface of the red cells. In later year had to test the hemagglutinin with red blood cells from different animals. Then in 1940 William C. Boyd tested the specificity hemagglutinating of lectin with human blood group that bring about to investigated on the structural basis of the specificity of the antigens related with the ABO blood group system.

Another activity of hemagglutinin are follow. Lectin usually found in defense or storage organism such as tuber, rhizome. Lectin is responsible in the plant's defense against different kinds of plant-eating organisms. In 1960 Peter C. Nowell who found the lectin in kidney bean *(Phaseolus vulgaris)* are mitogenie in that they stimulate the conversion of inactive lymphocytes into actively growing and dividing blast-like cells. But activity was repressed by a few mannose that assuring mitogenic activity is the effect of binding of lectins to sugars on the cell surface of the lymphocytes and was easy testing for a biological role of cell surface sugars. Additionally, lectin has the anticancer activity. Joseph C. Aub who finding this activity founded that wheat germ agglutinin (WGA) capability to specially

agglutinated malignant cells. It go to be investigator to changes in cell surface sugars that associated with the development of cancer cell.

2.4 Type of Lectin

2.4.1 Animal Lectin

Complex oligosaccharide structures are displayed at cell surfaces, incorporated into the extracellular matrix and attached to secreted glycoproteins. These oligosaccharides can serve structural roles, mediate movement of glycoconjugates to the cell surface or act as markers that mediate cell-cell and cell-matrix recognition events. The non-structural roles of sugars generally require the participation of sugar-binding lectins, in which sugar-binding activity can usually be ascribed to a single protein module within the lectin polypeptide. Such a module is referred to a carbohydrate-recognition domain (CRD). Changes have been introduced into the Ca^{2+} dependent carbohydrate recognition domain (CRD) of rat serum mannose-binding protein by site-directed mutagenesis to model the binding its of homologous galactose binding CRDs. Binding assays reveal that galactose binding activity nearly identical to that of the CRD from the asialoglycoprotein receptor can be introduced into the mannose-binding site by 3 single amino acid changes and insertion of a segment of 5 amino acids (Iobst *et al*., 1994). A lectin was isolated from the homogenate of the tunicate *Polyandrocarpa misakiensis* is a lectin is a monomeric protein with a molecular mass of 15 kDa and bound to an immobilized D-galactose column in the presence of calcium ion. The amino acid sequence of Polyandrocarpa lectin shows about 20-30s homology with those of fly, barnacle, sea urchin, and several vertebrate lectins that belong to C-type lectin (Suzuki *et al*., 1990). VIP36 was isolated from MDCK cells as a component of glycolipid-enriched detergent-insoluble complexes. The protein is localized to the Golgi apparatus and the cell surface, and belongs to a new family of legume lectin homologues in the animal secretory pathway that might be involved in the trafficking of glycoproteins, glycolipids or both. This binding requires Ca^{2+} and can be specifically inhibited by *N*-acetyl-D-galactosamine (Fiedler *et al*., 1996). The intact from baby hamster kidney lectin CBP30 contains 245 amino acid residues, including the initiating methionine residue, and is closely homologous to mammalian S-type lectins of similar size characterized in human, rat, and mouse species. The carboxyl terminal domain contains the carbohydrate binding

activity and the amino-terminal domain, which is extremely sensitive to bacterial collagenase, contains a repetitive sequence rich in glycine, tyrosine, and praline (Mehul *et al.*, 1994).

2.4.2 Plant Lectin

Plant lectins are being studied for over a century. Until a decade ago, most information was obtained from biochemical, molecular, and structural studies of a reasonably high but still limited number of abundant lectins from seeds and vegetative storage organs. In 1888 Stillmark founded the toxicity of castor bean extracts is linked to the presence of a proteinaceoushemagglutinating factor called 'ricin'. In 1898, Elfstrand introduced the term 'hemagglutinin' as a common name for all plant proteins that clump blood cells. In 1907 Landsteiner and Raubitschek reported for the first time the presence of nontoxic lectins in the legumes *Phaseolus vulgaris*, *Pisum sativum*, *Lens culinaris*, and *Vicia sativa.* The discovery that some hemagglutinins selectively agglutinated erythrocytes of a particular human blood group within the ABO system can be considered a milestone in the history of plant lectins. Once the blood group specificity of lectins was established, research aiming at the elucidation of the underlying mechanism for this biological activity was started. Only in 1952 it was shown that the agglutination properties of lectins are based on a specific sugar binding activity. Classical methods to study the interaction between lectins and carbohydrates often relied on the agglutination of erythrocytes or other cell types. The carbohydrate specificity of lectins was explored by indirect methods, such as for example, inhibition of precipitation or cellular agglutination by hapten sugars or glycoconjugates.More recently, the introduction of high performing techniques such as frontal aYnity chromatography and glycan microarrays enabled high throughput screening of large collections of carbohydrates and more complex glycans with only small amounts of a purified lectin.

2.4.3 Microoraganism

2.4.3.1 Fungi

The lectins in mushrooms are *Agaricus* species, *Amanita pantherina*, *Boletus satanas*, *Coprinus cinereus*, *Ganoderma lucidum*, *Flammulina velutipes*, *Grifola frondosa*, *Hericium erinaceum*, *Ischnoderma resinosum*, *Lactarius deterrimus*,

Laetiporus sulphureus, *Tricholoma mongolicum* and *Volvariella volvacea* exhibited a diversity of chemical characteristics. Some of them are monomeric, whereas others are dimeric, trimeric or tetrameric. Their molecular weights range from 12 to 190 kDa, and the sugar contents from 0 to 18%. Carbohydrate specificities involve mainly galactose, lactose and *N*-acetylgalactosamine. A small number of mushroom lectins are specific for fucose, raffinose, *N*-glycolyneuraminic acid and *N*-acetyl-D-lactosamine. Also, some of them showed antitumour/cytotoxic activities (Wang *et al*., 1998). A new lectin from the fungus. *Rhizopus stolonifer* agglutinates rabbit red blood cells and it activity was very stable, since it was insensitive to heat treatment at 70 °C for 10 min. *Rhizopus stolonifer* lectin recognizes the core structure of *N*-glycans with α(1–6)-linked L-fucose residues (Oda *et al*., 2003). A novel lectin from plant pathogenic fungus *Sclerotium rolfsii* showing the necessary factor to agglutination are protein and 1,3-β-glucan. This agglutinin specifically induced mycoparasitic behaviour in *Trichoderma harzianum* (Inbar *et al*., 1994).

2.4.3.2 Bacteria

The combining sites of type 1 fimbrial lectins of various species of enterobacteria were studied by measuring the inhibitory activity of linear and branched oligosaccharides and several glycosides of D-mannose on the agglutination of yeast cells by the organisms. The results showed that all five strains of *E. coli* tested possessed an elongated combining site best fitting a trisaccharide and including a hydrophobic region. The combining sites of *E. cloacae* and *E. agglomerans* were different from each other and from those of *Salmonella sp*. and E. coli. The results suggest that although classified under the general term mannose-specific, bacterial lectins in the form of type 1 fimbriae on different genera exhibit differences in sugar specificities (Firon *et al*., 1984). A total of 393 clinical bacterial isolates were tested for their ability to agglutinate yeast cells of either *Saccharomyces cerevisiae* or *Candida albicans*. Among the various strains tested, all isolates of *Serratia marcescens*, *Proteus morganii*, and *Citrobacter diversus*, as well as 94% of *Klebsiella pneumontae*, were positive. On the other hand, only 46% of the *Escherichia coli*, 48% of the salmonellae, 44% of the *Citrobacter freundii*, and 71% of the *Aeromonas hydrophila* isolates were positive. A quantitative determination of the lectin activity done by observing the agglutination of yeasts in microtiter plates showed that *S.*

marcescens isolates were the most avid binders to the yeast, whereas *Klebsiella* and *Citrobacter* isolates were the weakest (Mirelman *et al.*, 1980).

2.4.3.3 Algae

Twelve kinds of lectins isolated from four species of marine algae, *Boodlea coacta* (Chlorophyta) and *Hypnea japonica, Carpopeltis flabellata* and *Solieria robusta* (Rhodophyta), were compared for their chemical and biological properties. These lectins were proteins or glycoproteins, similar to terrestrial plant lectins. However, unlike most terrestrial plant lectins, they had a small molecular size (4,200 to 25,000 daltons), were mostly monomeric, and had no affinity for monosaccharides. They strongly agglutinated trypsin-treated rabbit erythrocytes, and their activities commonly were inhibited by glycoproteins bearing *N*-glycans (Hori *et al.*, 1990). A lectin from the red marine alga *Ptilota filicina* (PFL) agglutinated native and papain-treated human erythrocytes with preference for type O erythrocytes. It was inhibited by galactose and its derivatives. The most potent inhibitors were *p*-Nitrophenyl-*N*-acetyl-α-and β-D-galactosaminide. PFL was shown to be rich in acidic and hydroxyl amino acids but low in basic amino acids (Sampaio *et al.*, 1998).

2.5 Structure of Lectin

Different lectin families are in general structurally unrelated. And even in those cases where a common fold is recruited, convergent evolution is the most likely explanation. Some lectin families such as the galectins recognize only one specific oligosaccharide, and consequently have a very conserved recognition site. On the other extreme, members of the C type lectin family span a wide variety of specificities. Consequently, their recognition sites are highly variable, and different specificities can easily be engineered by site directed mutagenesis (Iobst *et al.*;1994; Kolatkar *et al.*, 1996). A general feature of binding sites of all lectins seems to be that they consist of a primary binding site that is capable of recognizing in a specific way a single monosaccharide residue, usually with a low affinity (in the millimolar range). Very often, but not always, there are further subsites that can be occupied by sugar residues connected to the one bound in the primary site. This allows for a modest increase in affinity.

Folding in common between plant and animal lectins are β-sandwich fold, β-Trefoil folds and Hevein domains. The legume lectin-like b-sandwich fold found in Galectins that conserved family of β-galactosyl binding lectins that occur in both vertebrates and invertebrates (Hirabayashi *et al*., 2002). Except for the legume lectins, galectins and pentraxins, it is observed in a number of carbohydrate processing and other enzymes such as β-glucanase and asparagine amidase (Keitel *et al*., 1993; Kuhn *et al*. 1994)
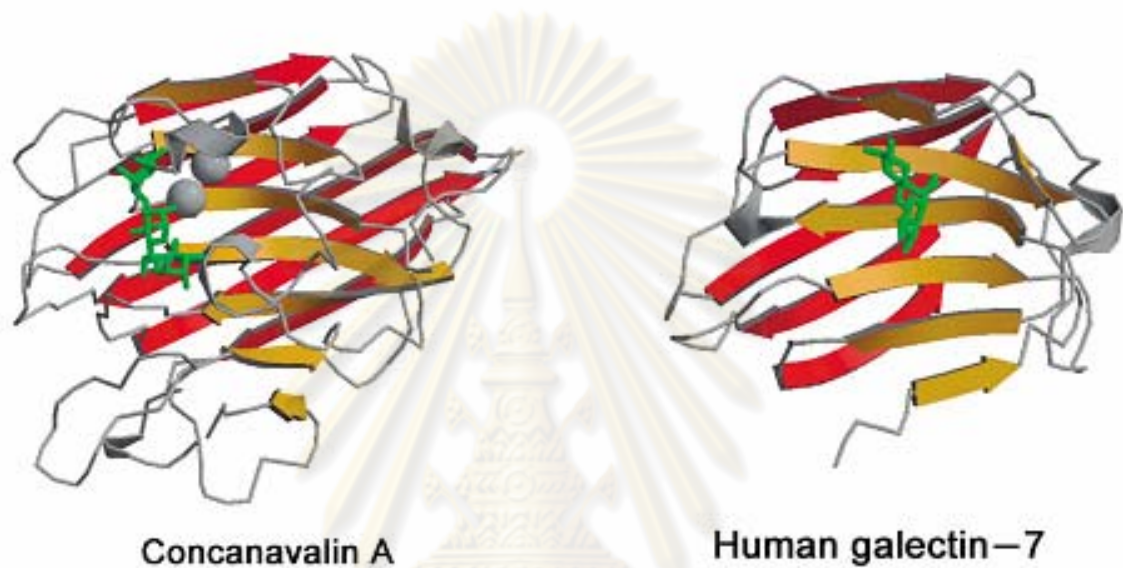
Figure 2.2 The legume lectin-like h-sandwich folds illustrated by a member of the galectins (human galectin-7 in complex with galactose) and one of the legume lectins (concanavalin A in complex with the trisaccharide (Man (α1-3)) Man (α1-6) Man).

The β-trefoil fold was first identified as a carbohydrate recognition domain in ricin (Montfort *et al*., 1987). Later, it was also found to be the fold of amaranthin. The β-trefoil fold is another fairly common fold, first identified in soybean trypsin inhibitor (Sweet *et al*., 1974). It consists of a repeat of three subdomains, each consisting of a fourstranded antiparallel β-sheet.
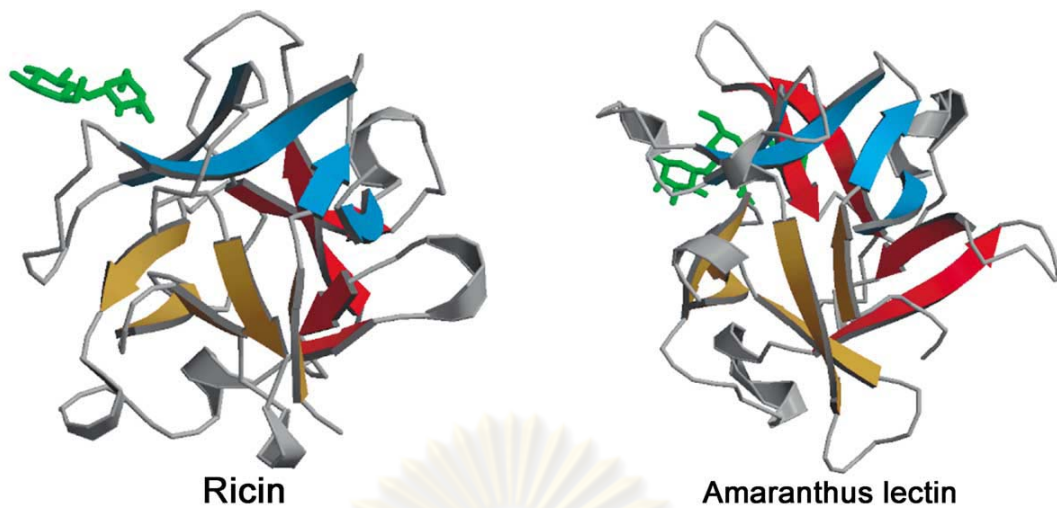
Figure 2.3 The h-trefoil folds illustrated by the first domain of the B-subunit ricin (in complex with lactose), the first domain of amaranthin (in complex with the Tantigen).

Hevein domains are a third case of convergent evolution of animal and plant lectin folds are the hevein domains. Hevein domains are small (30-45 amino acids) disulfide-rich domains that are found in many plant lectins and chitinases.
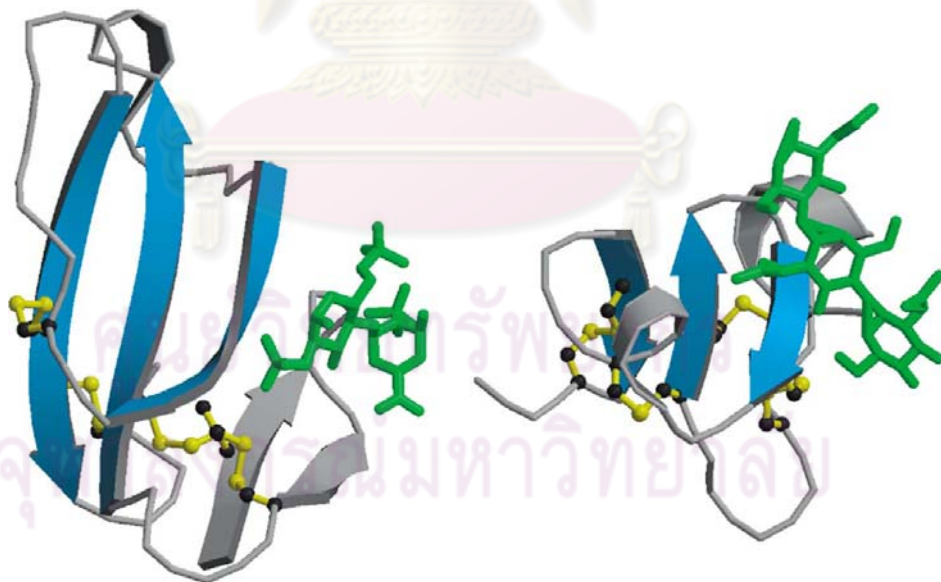
Figure 2.4 The hevein folds illustrated by cobra cardiotoxin and the first domain of nettle lectin. Chitotriose bound to the nettle lectin domain is shown in green ball-and-stick.

Lectins comprise a structurally very diverse class of proteins characterized by their ability to bind carbohydrates with considerable specificity. Although lectins bind monosaccharides rather weakly, they employ common strategies for enhancing both the affinity and specificity of their interactions for more complex carbohydrate ligands. Members of the legume lectin family show considerable sequence and structural homology, but differences in their carbohydrate-binding specificity. The legume lectin monomer has a molecular weight of 25,000 and is composed primarily of a six- and a seven-stranded antiparallel-sheet. Concanavalin A (Con A), *Lathyrus ochrus* isolectin I (LOL I), and pea lectin all show mannose and glucose-binding specificity, and the X-ray crystal structures of their carbohydrate complexes show a monosaccharide binding- site geometry very similar to that of the LOL I-α-methyl-o-mannopyranoside complex.

The first lectin structures to be determined derived from two phylogenetically conserved families, the *Leguminoseae* (Sharon and Lis 1990; Young and Oomen 1992) and the *Gramineae* (Raikhel *et al*., 1993) . Over the past ten years, major advances in X-ray crystallographic technology and the relative ease of isolation and crystallization of plant lectins have led to a rapid increase in crystal structures. The leguminous lectins clearly have dominated the field with some ten structures known today. Four of these, peanut lectin, soybean lectin, lentil lectin and hemagglutinin L, have been determined within the past three years. These lectins display diverse sugar-binding specificities structures.

Lectins of the *Amaryllidaceae, Orchidaceae, Alliaceae, Araceae* and *Liliaceae* families are in the class of the mannosespecific *Liliatae* and constitute the third major structurally characterized plant lectin superfamily (Van Damme *et al*., 1992). They are nonseed lectins of multigene families isolated from plant bulbs *(*Van Damme *et al*., 1993) and function either as dimers or tetramers, as do the legume lectins. Their strict and exclusive specificity solely for mannose has imparted some unusual biological properties *in vitro* to this lectin family including their antiviral properties against retroviruses (e.g. HIV) (Hammaar et al., 1994). Snowdrop lectin (GNA) is a tetrameric lectin (M_r = 50,000) and is the first member of the *Amaryllidaceae* family crystallographically investigated.

2.6 Biological activities of lectins

2.6.1 Cell agglutination

Lectin was disclosed that can able to cell-agglutinating and and sugar-specific protein. They showed to occur widely in plants and to some extent also in invertebrates. Toward the end of the 19th century, evidence started to accumulate for the presence in nature of proteins possessing the ability to agglutinate erythrocytes. Such proteins were referred to as hemagglutinins, or phytoagglutinins, because they were originally found in extracts of plants. In the first time that founded hemagglutinin was also highly toxic, was isolated by Stillmark from seeds of the castor tree (*Ricinus communis*) and was named ricin. By studying the inhibitory effect of the anti-ricin immune serum on the agglutinating activity of ricin, which was a quantitative relationship between the amount of antiserum and that of antigen it could neutralize and on this basis performed the first quantitative determination of an antibody *in vitro*. These studies thus demonstrated the specificity of the antibody response, the phenomenon of immunological memory, and the transfer of humoral immunity from a mother to its offspring.

Table 2.1 Functions of lectins

Lectin	Role
Microorganisms	
Amoeba	Infection
Bacteria	Infection
Influenza virus	Infection
Plants	
Various	Defense
Legumes	Symbiosis with nitrogen-fixing bacteria
Animals	
Calnexin, calreticulin, ERGIC-53	Control of glycoprotein biosynthesis
Collectins	Innate immunity
Dectin-1	Innate immunity
Galectins	Regulation of cell growth and apoptosis; regulation of the cell cycle; modulation of cell-cell and cell-substratum interaction
Macrophage mannose receptor	Innate immunity; clearance of sulfated glycoprotein hormone
Man-6-P receptors	Targeting of lysosomal enzymes
L-selectin	Lymphocyte homing
E- and P-selectins	Leukocyte trafficking to sites of inflammation
Siglecs	Cell=cell interactions in the immune and neural system
Spermadhesin	Sperm-egg interaction

2.6.2 Hemagglutinating activity

The Hemagglutination Assay (HA) is a quantification of lectin by hemagglutination. Some plant lectin families have surface that are able to agglutinate (stick to) human or animal Red blood cells (RBC) and bind to its *N*-acetylneuraminic acid. It is an easy, simple and rapid method and can be applied to large amounts of samples. The detailed conditions depend on the type of lectin. Some lectin binds RBCs only at certain pH values, others at certain ionic strengths. A lectin dilution (eg. 2-fold from 1:4 to 1:4096) will be applied to an RBC dilution (eg. 0.1% to 0.7% in steps of 0.2%) for approx. 30 min, often at 4 °C, because else viruses with neuraminidase activity will detach the lectin surface from the RBCs. Then the lattice forming parts will be counted and the titre calculated.
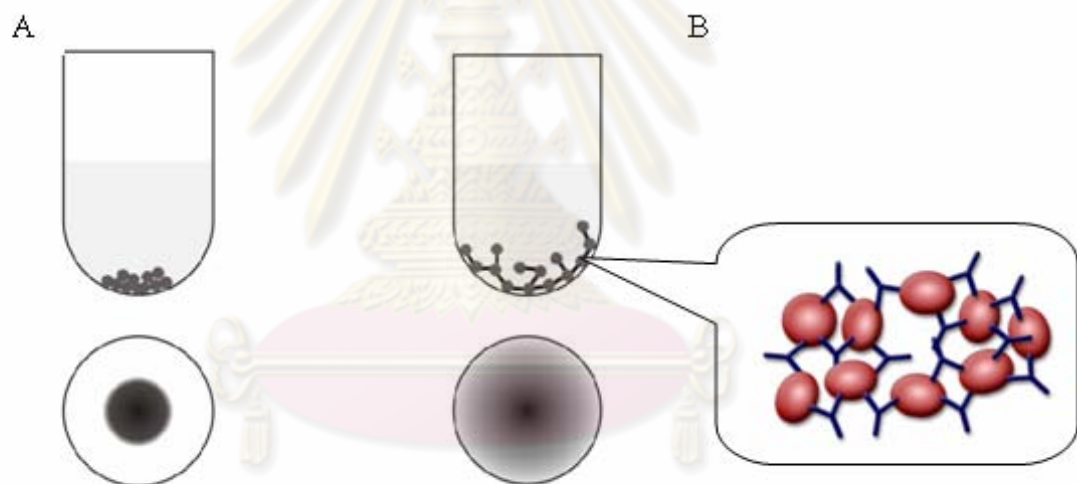
Figure 2.5 showed the hemagglution activity of lectin with Red blood cells (RBC). (A) Lectin or protein showed absent hemagglutination activity. The Red blood cells not be able to established the lattice and be like to the botton. (B) Lectin or protein showed present hemagglutination activity and red blood cell able to established the lattice.

HU: Hemagglutination unit (titer) defined as the reciprocal of the highest dilution exhibiting hemagglutination.

HU/mg: Specific activity is the number of hemagglutinating units per milligram of protein.

$$HU = 1/\text{lowest concentration}$$
$$= 2^n/ \text{ initial concentration}$$
$$\text{Specific activity} = HU/mg \text{ protein}$$

Agglutination of red blood cells, often used to test for the presence of antibodies directed against red cell surface antigens or carbohydrate binding proteins or viruses in a solution. Requires that the agglutinin has at least two binding sites phase. Crude proteins obtained by Mg/NP-40 extraction from Thai medicinal plants of the Curcuma species such as *C. longa*, *C. zedoaria*, *C. parviflora*, *C. amarissima*, *C. aeruginosa, C. aromatica, C. xanthorhiza*, *C. sparganifolia*, Khamindum, Haroynang, Maleung, Saligalinthong, Payanwan, Enleung and Khantamala exhibited agglutination activity against rabbit erythrocytes. A crude extract from Salingalinthong, a Thai Curcuma specie, exhibited the strongest hemagglutinating activity, 2×10^{-5} mg/ml. In another plants such as Con C or a lectin from *Canavalia cathartica* agglutinated A, B and O groups of human blood (Suseelan et al., 2007), *Vicia tetrasperma* (L.) Schreber is a new mannose glucose specific isolectin that showed higher specificity for rabbit RBC than human RBC, though it showed unspecificity with respect to the different human blood groups (Kunjeshwori Devi et al., 2009). *Agrocybe cylindracea* Lectin was purified from fruiting bodies of the mushroom *A. cylindracea* which agglutinated both rabbit and human erythrocytes (Liu et al., 2007). Since plant lectins reported were characterized upon their hemagglutination activities, it is possible that some plant which were inactived on hemagglutination

2.6.3 Carbohydrate specificity

In 1919, it the first time that obtained a pure hemagglutinin is concanavalin A and isolated from *Canavalia ensiformis*. The hemagglutination by concanavalin A was inhibited by sucrose, demonstrating for the first time the sugar specificity of lectins. It suggested that the hemagglutination induced by concanavalin A might be a consequence of a reaction of the plant protein with carbohydrates on the surface of the

red cells. A D-galactose-binding leaf lectin from *Erythrina indica* showed carbohydrate specificity directed towards D-galactose and its derivatives with pronounced preference for lactose (Emadeldin et al., 2002).

2.6.4 Antifungal activity

An antifungal activity is a medication used to treat fungal infections such as athlete's foot, ringworm, candidiasis (thrush), serious systemic infections such as cryptococcal meningitis, and others. Because of antifungal activity is an essential activity of lectin therefore it not surprise which can founded these activity in plant, animal or microbial that have lectin in it organelle. In *Curcuma zedoaria* and *Curcuma malabarica* tubers that plants in Zingoberaceae family showed antifunal activity to *Candida albicans* at a concentration of 75 µg/well (Wilson *et al*., 2005). A lectin from *Myracrodruon urundeuva* heartwood exhibited antifungal Cercobin in growth inhibition of phytopathogenic fungi (Roberto *et al*., 2009). Castamollin which the protein was isolated from Chinese chestnut (*Castanea mollisima*) seeds displayed antifungal activity against *Botrytis cinerea*, *Mycosphaerella arachidicola*, *Physalospora piricola*, and *Coprinus comatus* but was devoid of lectin activity (Wang et al., 2001).

2.6.5 Antimicrobial activity

Minimum inhibitory concentration (MIC) of an antibacterial is defined as the maximum dilution of the product that will still inhibit the growth of a test microorganism. In several plant which have been lectin mainly have the antimicrobial activity. Zingiberaceae plant extracts exhibited antimicrobial activity against all tested food microorganisms. *Hedychium* and *Vanoverberghia*, did not show antimicrobial activities on *Escherichia coli* and *Vibrio parahaemolyticus* (Chen et al., 2008). In *Araucaria angustifolia* seeds lectin showed significant antimicrobial activity, mainly against Gram-positive bacteria, and anti-inflammatory effect in acute cellular inflammation. Amphibians respond to microbial infection through cellular and humoral defense mechanisms such as antimicrobial protein secretion. Two β-galactoside-binding lectins which isolated from the skin of *Bufo arenarum* showed strong bacteriostatic activity against Gram negative bacteria (*E. coli* K12 4100 and wild strains of *E. coli* and *Proteus morganii*) and Gram positive bacteria (*Enterococcus faecalis*) (Riera *et al*., 2003). The antibacterial activity of these lectins

may provide an effective defense against invading microbes in the amphibian *Bufo arenarum.*
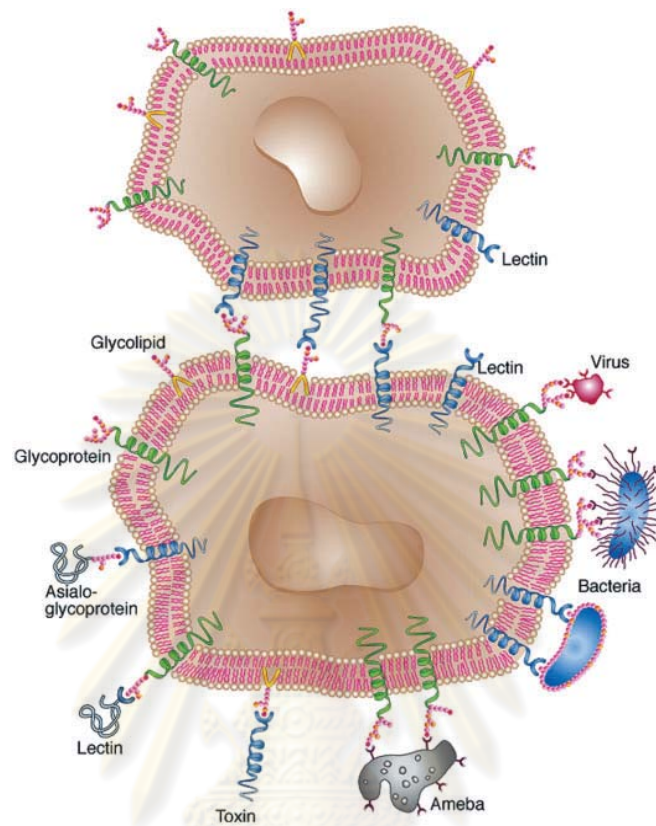
Figure 2.6 Cell surface lectin–carbohydrate interactions. Lectins serve as means of attachment of different kinds of cell as well as viruses to other cells via the surface carbohydrates of the latter. In some cases, cellsurface lectins bind particular glycoproteins (e.g., asialoglycoproteins), whereas in other cases the carbohydrates of cell surface glycoproteins or glycolipids serve as sites of attachment for biologically active molecules that themselves are lectins (e.g. carbohydrate-specific bacterial and plant toxins, or galectins)

2.6.6 Alpha-glucosidase inhibition activity

Alpha-glucosidase inhibitor is a class of oral medications for type 2 diabetes that decrease the absorption of carbohydrates from the intestine, resulting in a slower and lower rise in blood glucose throughout the day, especially right after meals. Before carbohydrates are absorbed from food, they must be broken down into smaller

sugar particles like glucose by enzymes in the small intestine. One of the enzymes involved in breaking down carbohydrates is called alpha glucosidase. By inhibiting this enzyme, carbohydrates are not broken down as efficiently and glucose absorption is delayed. The alpha-glucosidase inhibitors include Precose (acarbose) and Glyset (miglitol).
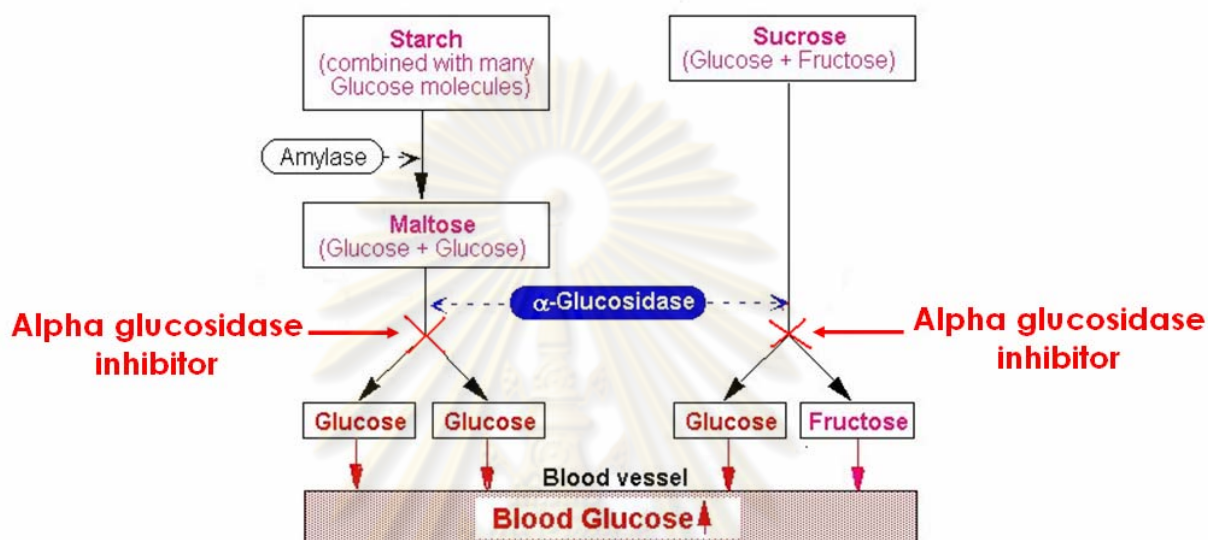
Figure 2.7 Digestion of Carbohydrate (starch and sucrose) in the body

Natural curcumin (1), demethoxycurcumin (2) and bisdemethoxycurcumin (3) isolated from *Curcuma longa* (turmeric) which plant in Zingiberaceae family, and synthetic curcumin analogs (A_{1-7}, B_{1-7}, C_{1-6} and D_{1-7}) were evaluated in vitro for the α-glucosidase inhibitory activity via UV and circular dichroism (CD). inhibitory effect with IC_{50} of 23.0 μM, and the synthetic compounds A_2, B_2, C_2 and D_2 showed potent inhibitory effects with IC_{50} of 2.8, 2.6, 1.6 and 8.2 μM, respectively (Du et al., 2006).

In another plants, *Morus Alba* leaves were tested (in-vitro) for alpha glucosidase inhibitory activity. *Morus Alba* showed potent activity with an IC_{50} value of 28.11μg/ml (Yogisha and Raveesha 2009). *Scutellaria baicalensis*, *Rheum officinale*, and *Paeonia suffruticosa* which extracted with methanol showed potent inhibitory activity against rat intestinal sucrase. The active principles were identified as baicalein from the first and methyl gallate from the last two plants. In addition to its activity against the rat enzyme, baicalein also inhibited human intestinal sucrase

expressed in Caco-2 cells(Nishioka *et al*., 1998). Alpha glucosidase inhibitor that can purified from red alga *Grateloupia elliptica* cnotain IC_{50} values of 2,4,6 tribromophenol and 2,4-dibromophenol were 60.3 and 110.4 μM against *Saccharomyces cerevisiae* α-glucosidase, and 130.3 and 230.3 μM against *Bacillus stearothermophilus* α-glucosidase, respectively (Kim *et al*., 2008). Nepalese medicinal herb Pakhanbhed (*Bergenia ciliata*, Haw.) exhibited inhibitory activities against rat intestinal a-glucosidase and porcine pancreatic a-amylase (Bhandari *et al*. 2008). In *Spiraea cantoniensis* Flower which including flavonol caffeoylglycosides also showed α-glucosidase-inhibitory activity(Yoshida *et al*., 2008). The compound of *Andrographis paniculata* was a very potent inhibitor against α-glucosidase with an IC_{50} value of 6 μM including 15-*p* chlorobenzylidene-14-deoxy-11, 12-didehydro-3, 19-dinicotinateandrographolide (Xu *et al*., 2007).

2.7 Potential applications of lectins

2.7.1 Antiproliferative activity and cytotoxicity

Two lectins, TML-1 and TML-2, were isolated from *Tricholoma mongolicum* showing hemagglutinating activities and sensitive to lactose inhibition and are stable between 10 and 80 °C. They exhibit antiproliferative activities against mouse monocyte-macrophage PU5-1.8 cells and mouse mastocytoma P815 cells *in vitro* (Wang et al., 1995). A novel lectin having specificity towards a complex glycoprotein asialofetuin was purified from tubers of *Arisaema flavum* (Schott.). *A. flavum* lectin showed potent mitogenic activity towards BALB/c splenocytes and human lymphocytes in comparison to Con A, a well-known plant mitogen. AFL also showed significant in vitro antiproliferative activity towards J774 and P388D1 murine cancer cell lines (Singh et al., 2004).

2.7.2 Biocontrolling agents

Snowdrop lectin (*Galanthus nivalis* agglutinin, GNA) has previously been shown to confer significant levels of protection against the lepidopteran pest *Lacanobia oleracea* when expressed in transgenic potato. The effect of GNA on the parasitism of *L. oleracea* by the gregarious ectoparasitoid *Eulophus pennicornis* was investigated. Maize-based, and potato leaf-based diets containing GNA, and excised

transgenic potato leaves expressing GNA, were fed to *L. oleracea* larvae from the beginning of either the third or fourth larval instar (Bell et al., 1998). When the mycoparasitic, biocontrol fungus Trichoderma harzianum was allowed to grow on nylon fibers treated with concanavalin A or Scierotium rolfsii lectin, it coiled around the nylon fibers and produced hooks in a pattern similar to that observed with the real host hyphae. The incidence of interaction between *T.harzianum* and *S. rolfsii* lectin-treated fibers was significantly higher than that of the controls (Inbar and Chet 1992).

2.7.3 Lectin derivatives preparation

A stable, biologically active glycosylated insulin derivative that is complementary to the major combining site of concanavalin A has been synthesized. Hormone release is proportional to the quantity of glucose present. Glucose regulation of exogenous insulin delivery could have important applications in the therapy of diabetes mellitus (Brownlee and Cerami 1979). The derivatives of polysaccharides are prepared by activation of the polysaccharide with cyanogen bromide and subsequent reaction with fluoresceinamine. The fluorescein-derivatized polysaccharides were found to have the same potency in inhibiting lectin-mediated hemagglutination as the underivatized polysaccharide (Glabe *et al*., 1983).

2.7.4 Bone marrow transplantation

Mouse bone marrow and spleen cells were fractionated with the aid of soybean agglutinin and peanut agglutinin. A test for spleen colony-forming units in the isolated fractions showed that the hemopoietic stem cells are agglutinated by both of these lectins. The capacity of the agglutinated fractions to reconstitute lethally irradiat alogeneic mice was investigated. A sequential fractionation of'splenocy'tes from SWR donors by soybean agglutinin and peanut agglutinin, or a single fractionation by soybean agglutinin of splenocytes from BALB/c donors, afforded a cell fraction that successfully reconstituted lethally irradiated (BALB/c X C57BL/6)F1 mice, without complications due to graft-versus-host reaction (Reisner et al., 1978).

2.7.5 Blood Typing

A blood group B-specific lectin from the mushroom *Marasmius oreades* (MOA) are strongly precipitated blood group type B substance, was nonreactive with type A substance, and reacted weakly with type H substance. MOA also reacts strongly with murine laminin from the Engelbreth-Holm-Swarm sarcoma and bovine thyroglobulin, both of which contain multiple Galα1,3Galβ1,4GlcNAc end groups (Winter *et al*., 2002). Using a radioimmunoassay to measure the relative potencies of a wide range of chemically modified structures related to the H-type 2 human blood group determinant, evidence was accumulated that the binding of αLFuc(l→2) βDGal(1→4)- βD GIcNAc-OMe by the lectin I of *Ulex europaeus* involves a wedge-shaped amphiphilic surface which extends on one side of the molecule from the methoxy aglycon to OH-3 of the βD Gal unit (Oleh et al., 1985).

2.8 Free energy of lectin

Free energy is a state function because it is formally defined only in terms of state functions, the state functions enthalpy and entropy, and the state variable temperature. The definition of free energy is

$$G = H - TS$$

In this equation H is the enthalpy, S is the entropy, and T is the absolute temperature.

A change in free energy dG would then be given by dG = dH - d(TS). Since temperature is a state variable and entropy is a state function they are divisible, and so dG = dH - TdS - SdT. However, at constant temperature the change in temperature dT is zero, so the term SdT is zero also. For any constant-temperature (isothermal) change, then dG = dH - TdS. Rearrangement of this equation gives, for larger changes, the Gibbs-Helmholtz equation:

$$\Delta H = \Delta G + T\Delta S$$

TdS is an energy, the energy not available for doing work. The equation above therefore has the physical meaning total energy available as heat = ΔG + energy not available for doing work

ΔG must be the energy which is available for doing work. This free or available energy which can do useful work is then a most useful thing to know or to have.

The conventions of heat flow q are also those of enthalpy change. When ΔH is negative, heat is evolved (or given off) in the exothermic process or reaction, while when ΔH is positive, heat is absorbed in the endothermic process or reaction from the surroundings. Free energy change also follows the same conventions. When ΔG is negative, energy is evolved (given up) in the process or reaction and the change can do useful work on something else. This is a spontaneous process, and is sometimes called an exergonic (energy-giving) process. When ΔG is positive, energy is absorbed in the process or reaction and useful work must be done on the materials so that the reaction will occur. This is a nonspontaneous process, and is sometimes called an endergonic (energy-requiring) process.

The thermal stability of β-lactoglobulin (β-Lg) dimer was reassessed base on three stage denaturation process involving dissociation and unfolding (dimers ↔ monomer ↔ unfolde state). The Gibb free energy change for β-Lg dissociation unfolding was 56.7 kJmol^{-1} compared with an estimated was 14 kJmol^{-1} with β-Lg monomer as the reference native state (Apenten *et al*., 2002).The unfolding pathway of two very similar tetrameric legume lectins soybean agglutinin (SBA) and Concanavalin A (ConA) were determined using GdnCl-induced denaturation. Both proteins displayed a reversible two-state unfolding mechanism. It was found that the ΔG of unfolding of SBA was much higher than ConA at all the temperatures at which the experiments were done. The higher conformational stability of SBA in comparison to ConA is largely due to substantial differences in their degrees of subunit interactions. Ionic interactions at the interface of the two proteins especially at the noncanonical interface seem to play a significant role in the observed stability differences between these two proteins (Sinha *et al*., 2005). The conformational stability of the homodimeric pea lectin was determined by both isothermal urea-induced and thermal denaturation in the absence and presence of urea. The conformational stability (ΔGs) and the ΔCp for the protein unfolding is quite high, at about 18.79 kcal/ mol and 5.32 kcal/(mol K), respectively, that may be a reflection of the relatively larger size of the dimeric molecule (M$_r$ 49,000) and, perhaps, a consequent larger buried hydrophobic core in the folded protein (Ahmad *et al*., 2004).

2.9 Thermostability lectin

Theoretical analysis and computational predictions of protein thermostability of thermal stabilization proteins are interested. Although comparison between mesophilic and thermophilic sequences has suggested some thermostabilization mechanisms. Two of the mechanisms used by nature are seen as the major factors governing thermostability. One is the electrostatic forces of charged amino acids within a protein and the packing of its hydrophobic core on the other. Other mechanisms that have also been implicated such as hydrogen bonding, α-helix stabilization, backbone rigidifying (Mozo-Villiarias and Querol 2006).

The hemagglutinin from *Phaseolus vulgaris* is a homotetrameric leucoagglutinating seed lectin. Its three dimensional structure showed similarity with other members of the legume lectin family. The tetrameric form of this lectin is pH dependent. The lectin was found to be extremely thermostable with a transition temperature around 82 ºC and above 100 ºC at pH 2.5 and 7.2 respectively (Biswas and Kayastha Arvind 2002). A lectin from *Erythrina speciosa* seeds is the thermostability lectin**.** At 65 °C for more than 90 min the lectin was fairly stable; however, when heated at 70 °C for 10 min it lost more than 80% of its original activity and was totally inactivated at 80 °C for less than 10 min (Konozy *et al.*, 2003). The lectin from jackfruit *(Artocarpus integrifolia)* seeds is thermally stable and loses its activity above 75 °C. The hemagglutinating activity remains unchanged in the presence of bivalent cations*,* Ca^{2+},Mg^{+2}, Mn^{2+},e tc.It is a metalloprotein(Ahmed & Chatterjee 1989). A new lectin from seeds *Moringa oleifera* was investigated. It activities were carbohydrate inhibited by azocasein and asialofetuin abolished *Moringa oleifera* lectin hemagglutinating. The lectin was thermostable at 100 °C during 7 h (Santos Andrea *et al*., 2009).

2.10 Protein sequencing

2.10.1 Liquid chromatography – mass spectrometry (LC-MS/MS)

Liquid chromatography-mass spectrometry/mass spectrometry (LC-MS, or alternatively HPLC-MS/MS) is an analytical chemistry technique that combines the physical separation capabilities of liquid chromatography (or HPLC) with the mass analysis capabilities of mass spectrometry. LC-MS is a powerful technique used for many applications which has very high sensitivity and specificity. Generally its

application is oriented towards the specific detection and potential identification of chemicals in the presence of other chemicals (in a complex mixture).
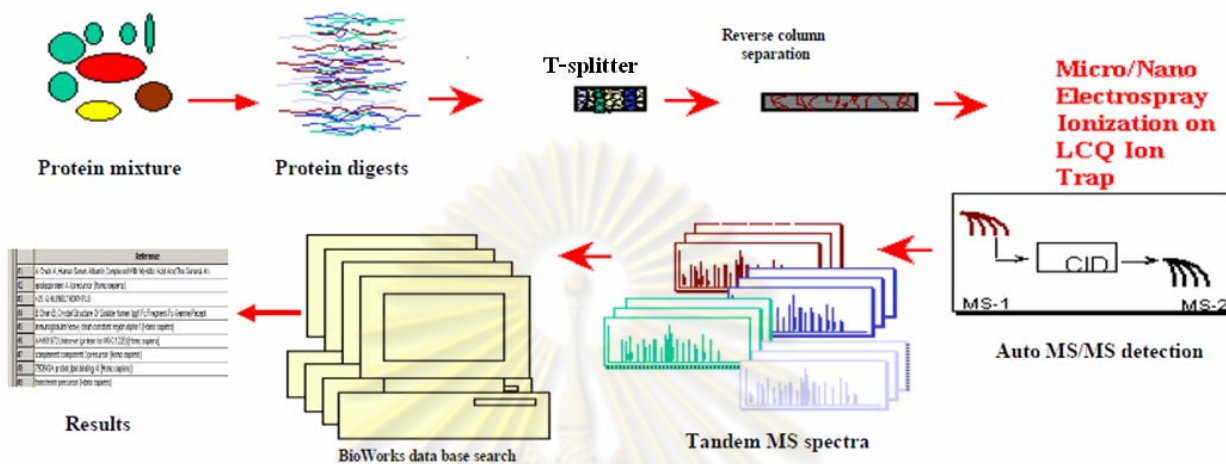
Figure 2.8 Show the process of protein identification with LC-MS/MS.

An on-line capillary LC-MS/MS system consists of conventional HPLC pumps, transfer tubing, a pre-column flow splitter, a liquid junction, a reverse –phase micro capillary column and a tendem mass spectrometer. The and mass spectrometer are control by same software to aloe coupling between chromatography and ion detection. The flow rate at conventional pump operate (∼100 μl/min) and the optimum flow rate for microcapillary column(∼300 μl/min) are vastly different. A flow restrictor (Tsplitter) permits the gradient to form quickly and be distributed to the microcapillary column and acceptable floe-rate. A conductive liquid junction (e.g. gold wire) to a high voltage source (1-2 kV) is needed to promote electrospray. Although numerous method of coupling liquid chromatography to MS have been explored, electrospray ionization has transform LC-MS/MS into a routine laboratory procedure sensitive enough to analyze peptide and protein at low concentration. Currently detection limit of a few femtomols of peptide material loaded on the column make this technique compatible with silver-strain, fluorescently labeled, or faintly strained Coomassie gel bands and capable of detecting proteins and peptides present at a low copy number per cell. In addition, the high resolution

chromatography step make this technique attractive for the analysis of peptide derived from complex mixtures which are reduced, denatured and alkylated prior to protiolytic digestion. The resulting peptide mixture is then lyophilized and resuspended in the LC buffer for analysis. A sample can be loaded and eluted on the capillary column in different ways depending on its peptide concentration and volume. Commonly, the sample is loaded on to a pre-column trap at µl flow-rate for concentration and fast desalting and then eluted to the reverse phase column for separation.

CHAPTER III

EXPERIMENTAL

3.1 Biological material

The fresh rhizomes of *K. parviflora* were purchased from a local market in Bangkok, Thailand. A voucher specimen (BK51772) is deposited at the Bangkok Herbarium of The Plant Variety Protection Division, Department of Agriculture, Bangkok, Thailand. Human blood samples (ABO system) were obtained from healthy donors at the Thai Red Cross Society, Bangkok, Thailand. All other non-human animal blood was supplied from the Division of Production and Supply, National Laboratory Animal Center, Mahidol University, Nakhon Pathom, Thailand. The three plant pathogenic fungal species (strains) used in the bioassays for lectin antifungal activity, *Collectotrichum cassicola* (DOAC 1196), *Exserohilum turcicum* (DOAC 0549) and *Fusarium oxysporum* (DOAC 1258), were obtained from the Division of Plant Disease and Microbiology, Department of Agriculture, Bangkok, Thailand. The four bacterial strains used in the bioassays for lectin antibacterial activity, *Bacillus subtilis* ATCC6633, *Staphylococcus aureus* ATCC 25923, *Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853, plus the diploid fungus (yeast), *Candida albicans* ATCC 10231, were obtained from the Division of Plant Disease and Microbiology, Department of Agriculture, Bangkok, Thailand.

3.2 Chemicals and reagents

Concanavalin A Sepharose (ConA Sepharose) and α-glucosidase from *Saccharomyces cerevisiae* were purchased from Sigma Chemicals Co. (USA). Sephacryl S-100 size exclusion column was purchased from Amersham Pharmacia Biotech (Uppsala, Sweden). Methyl-α-D-glucopyranoside was purchased from Fluka (Germany). The reagents used in SDS-PAGE were obtained from Plusone Pharmacia Biotech (Sweden), except for the low molecular weight marker protein calibration kit which was purchased from Amersham Pharmacia Biotech (UK). All other biochemicals and chemicals used in the investigation were of analytical grade.

3.3 Purification of lectin from rhizomes of *K. parviflora*

To purify the *K. parviflora* lectin, fresh rhizomes (1 kg wet weight) were washed, cut into small pieces and homogenized in a blender in TBS (20 mM Tris-HCl buffer, pH 7.4, containing 0.15 M NaCl) at 1.5 kg/L, and left to extract with stirring overnight at 4 °C. The insoluble material was removed by filtration through two layers of cheesecloth and centrifuged at 15,000 x g for 30 min at 4 °C. Proteins were then precipitated from the clear filtrate (a crude TBS-soluble rhizome extract) by the addition of ammonium sulfate to 80% saturation and stirring overnight at 4°C. The precipitate, mostly as a suspension, was then harvested by centrifugation at 15,000 x g for 30 min at 4 °C, the supernatant discarded and the pelleted proteins dissolved in TBS, and dialyzed against excess water. The dialzate was then submitted, at 10 mL of the preparation (~2 mg/mL) at a time, to affinity chromatography on a 1.6 x 20 cm column of ConA-Sepharose equilibrated and eluted with TBS at a flow rate of 60 mL h^{-1}. After elution of the unbound proteins in the equilibrium buffer (i.e. when the eluate A_{280} fell to <0.05), the adsorbed proteins were eluted with a increasing gradient of 0.5 M methyl α-D-glucopyranoside as the competitor, to desorb the lectin from the column and 10 mL fractions collected. The fractions containing hemagglutinating activity against rabbit erythrocytes (see below) were pooled, and dialyzed against TBS, and then submitted to gel filtration chromatography on a 1.6 x 60 cm Sephacryl S-100 column equilibrated and eluted with TBS at a flow rate of 30 mL h^{-1}, loading 5 mL (~1 mg/mL) at a time and collecting 5 mL fractions. Fractions were screened for hemagglutination activity from the column and contiguous positive fractions were pooled and dialyzed against an excess of water prior to further analysis.

3.4 Protein determination and carbohydrate content

The method described by Bradford (1976) was used with serial dilutions of a known concentration of bovine serum albumin (BSA) as standard. Absorbance at 280 nm was also used to determine the protein content of column eluates. Total neutral carbohydrate content was estimated by the method of Dubois *et al*. (1956) using D-glucose as standard.

3.5 Hemagglutination assays

In the hemagglutination activity assay, a serial twofold dilution of the lectin solution in microtiter U-plates (50 ml) was mixed with 50 ml of a 2% (v/v)

suspension of rabbit red blood cells in TBS at room temperature. The results were recorded after about 1 h when erythrocytes in the blank had fully sedimented. The hemagglutination titer, defined as the reciprocal of the highest dilution of the lectin solution exhibiting hemagglutination, was reckoned as one hemagglutination unit. Specific activity is the number of hemagglutination units per mg protein Hemagglutination activity was assayed separately against erythrocytes from rabbits, rats, mice, guinea pigs, geese, sheep and the four human ABO blood groups.

3.6 Molecular weight determination by reducing SDS-PAGE

Molecular mass estimation under denaturing conditions was carried out by discontinuous SDS-PAGE following Laemmli (1970) and using a 15% (w/v) polyacrylamide resolving gel. Samples (20 μg total protein) and standards were prepared in Tris-HCl buffer, pH 6.8, containing 2% (w/v) SDS and 5% (v/v) 2-mercaptoethanol, followed by heating at 100 °C for 10 min. The molecular mass markers used were: phosphorylase b (94.4 kDa), albumin (66.2 kDa), ovalbumin (45.0 kDa), carbonic anhydrase (31.0 kDa), trypsin inhibitor (21.5 kDa) and α-lactalbumin (14.4 kDa). Resolved gels were stained by the standard Coomassie R-250 blue method to visualize the protein bands.

3.7 Effect of temperature on lectin hemagglutinating activity and thermostability

The effect of temperature on the hemagglutinating activity of the lectin was determined by incubating lectin samples (pH 7) at temperatures from 30 to 95 °C, inclusive, in 5 °C increments, and at room temperature, for 30 min, followed by an immediate chill at 4 °C for 15 min. At least four replicates were done for each temperature treatment. Thereafter, an aliquot of each treated lectin solution was incubated at various temperatures (60, 70, 80 and 90 °C) and times (10, 20, 30, 60, 90, 120 and 150 min), thereafter the hemagglutinating activity was tested. The free energy change (ΔG) of activation of the lectin denaturation process was determined using the Arrhenius expression [22]. The velocity constant of the reaction (k_1) was first determined as the slope of the curve obtained by the expression:

$k_1 t = -\ln A/A_0$

where A = residual hemagglutinating activity after heat treatment, A_0 = initial hemagglutinating activity before heat treatment, and t = time of heat treatment (in seconds).

The velocity constant $k1$ is related to the standard free energy change by the following formula:

$$\Delta G = RT \ln(kT/k_1 h)$$

where R is the gas constant (1.987 cal mol^{-1} K^{-1}), T is the absolute temperature (K), k is the Boltzmann constant (1.37 x 10^{-6} erg K^{-1}), k_1 is the velocity constant, and h is Planck's constant (6.25 x 10^{-27} erg s^{-1}).

3.8 The pH-dependence of agglutination activity

The pH stability of the lectin was evaluated by incubating the lectin samples in the following buffers of essentially the same osmolarity but varying from pH 2 - 10 for 18 h at 25 ºC. Different buffers were used according to the desired pH, within the following pH ranges; 20 mM glycine-HCl buffer (pH 2.0 - 4.0), 20 mM sodium acetate buffer (pH 4.0 - 6.0), 20 mM potassium phosphate buffer (pH 6.0 - 8.0), 20 mM Tris-HCl buffer (pH 8 - 10) and 20 mM glycine-NaOH buffer (pH 10.0 - 12.0). The residual hemagglutinating activity was then assayed after adjusting the mixture back to pH 7.4 with the addition of a two-fold volume of TBS. The hemagglutination lectin titres attained were compared with the control which was set as 100%. At least three replicates were done for each test to confirm the results.

3.9 Effect of metal ions on the lectin's hemagglutination activity

The effect of six different divalent metal cations, or the selective chelating agent EDTA, on the lectin's hemagglutinating activity was monitored. The purified lectin (1 mg/ml) was incubated for 10 h with one of Ca^{2+}, Co^{2+}, Fe^{2+}, Hg^{2+}, Mg^{2+}, Mn^{2+} and EDTA, at various concentrations (12.5, 25, 50 and 100 mM) with continuous shaking. After that, 50 µl of a 2-4% (v/v) suspension of rabbit erythrocytes was added, and the hemagglutination was scored after 1 h as described above, using at least three replicates for each assay.

3.10 Internal amino acid sequence of lectin by LC/MS/MS

The internal amino acid sequence of the purified lectin from *C. amarissima* rhizomes was performed by in-gel trypsin digestion of the protein and sequencing of the different tryptic peptides by LC/MS/MS mass spectrometry. Coomassie-stained protein spot was excised from SDS-PAGE gel and washed with 3% hydrogenperoxide. The protein was in-gel reduced, alkalated and digested with trypsin. After digestion, the peptides were twice extracted from gel with 50% acetronitrile/0.1% TFA and air dried. The trypic peptides were subjected to LC-nano ESI-MS/MS. All collected LC/MS/MS data were processed and submitted to a MASCOT search of an inhouse NCBI database. The following criteria were used in the Mascot search: trypsin cleavage specificity with up to three missed cleavage, cysteine carbamidomethyl fixed modification, methionine oxidation variable modifications, ±0.2 Da peptide tolerance and MS/MS tolerance, and ESI-TRAP fragmentation scoring.

3.11 Assay of antifungal activity

The assay for antifungal activity towards the plant pathogenic fungi *E. turcicum, F. oxysporum* and *C. cassicola* was carried out in 90 x 15 mm petri-plates containing 10 ml potato dextrose agar (PDA). After the mycelial colony had developed, sterile blank paper disks (0.625 cm in diameter) were placed at a distance of 0.5 cm away from the rim of the mycelial colony. The suitably diluted lectin or control samples, dissolved in TBS, were added (10 μl) to the disks and the plates then incubated at 25 °C until mycelial growth had enveloped the peripheral edges of the control (TBS) disks and had formed crescents of inhibition around the paper disks containing the purified lectin samples in TBS. At this stage, the diameter of the clear zone of inhibition surrounding the sample was taken as a measure of the inhibitory power of the sample against the particular test organism.

3.12 Antibacterial activity test

The four bacterial strains, *E. coli, S. aureus, B. subtilis* and *P. aeruginosa*, plus the diploid fungi (yeast), *C. albicans*, were separately cultured in nutrient broth (NB) for the bacteria and yeast malt media (YM) for the yeast. A series of two-fold serial dilutions of the purified *K. parviflora* rhizome lectin solution were prepared in a total volume of 50 μl with sterile distilled water in each well of a 96 well plate. Each well

was then inoculated with 50 μl NB (bacteria) or YM (yeast) media and then 50 μl of the respective bacterial or yeast culture, prepared from a six hour old broth culture, and adjusted to a McFarland standard turbidity number 0.5, was added making a total volume in each well of 150 μl. Control wells had either no bacteria or yeast (but plain NB or YM media instead), or included ampicillin or penicillin separately at a final concentration of 1 mg/ml as positive controls to determine the sensitivity of tested solutions. The plate was covered with a sterile plate sealer and incubated for 24 hours at either 37 °C for the four bacterial species or at 30 °C for *C. albicans*, and then the absorbance at 600 nm was used to measure the cell densities in comparison with the negative (and positive) controls and standardized with the McFarland standard solutions. McFarland standards were used as a reference to adjust the turbidity of bacterial suspensions so that the number of bacteria will be within a given range.

3.13 Alpha-glucosidase inhibition activity

Alpha-glucosidase inhibition was assayed in 50 mM sodium acetate buffer at pH 5.5 with 1 mM *p*-nitrophenyl-α-D-glucopyranoside (PNPG) as the substrate. The enzyme (1 Unit/ml final concentration) was mixed with the purified lectin in 50 μl volume, and incubated at 37 °C for 10 min. Then 500 μl of 1 mM PNPG was added to initiate the enzyme reaction which was incubated at 37 °C for 30 min before being stopped by the addition of 1 ml of 1 M Na_2CO_3. Alpha-glucosidase activity was determined by measuring the release of the yellow *p*-nitrophenol at 400 nm. One unit of α-glucosidase is defined as the amount of enzyme liberating 1.0 μmole of *p*-nitrophenol (PNP) per minute under the conditions specified. 1 mM 1-deoxynorijimycin was used as the positive control in this study. The half maximal inhibition concentration (IC_{50}) of the lectin sample was determined by constructing a dose-response curve and from this determining the concentration of the lectin sample that inhibited 50% of the maximal α-glucosidase enzyme activity.

CHAPTER IV

RESULT AND DISCUSSION

4.1 Purification of *K. parviflora* lectin

This study reports *on the* purification and characterization of lectin from the black rhizomes of *K. parviflora*. The fresh rhizomes were diced, and then homogenized and extracted overnight in TBS before filtration and centrifugation to leave the clear supernatant, the crude TBS-soluble rhizome extract. Purification of *K. parviflora* lectin started with an 80% saturation ammonium sulfate precipitation of the crude TBS-soluble extract, which reduced the total protein level by 10% and resulted in a slight (1.04-fold) increase in the specific hemagglutination activity at the cost of a 6% loss of yield. Indeed, hemagglutinating activity (total and specific) was used as a surrogate lectin marker to monitor all the purification procedures. Following dialysis, ConA Sepharose affinity chromatography was employed. All the hemagglutinating activity remained in the bound fraction, with no detectable activity in the void elutant, but rather eluted in the presence of the competitor, 0.5 M methyl α-D-glucopyranoside (Figure 4.1), resulting in a further decrease in the total protein of ~99% and the effective loss of the purple pigments, with a further 72-fold purification at a cost of a further 20% reduction in the lectin yield (Table 4.1). The recovered ConA Sepharose bound fraction with hemagglutinating activity was dialyzed, concentrated and then subjected to Sephacryl S-100 gel filtration chromatography, with the protein elution profile shown in Figure 4.2. Only one peak of protein eluted from the column and this final *(*potentially homogeneous) lectin preparation was purified by 89.8-fold with a 40% yield relative to the starting crude extract (Table 4.1).
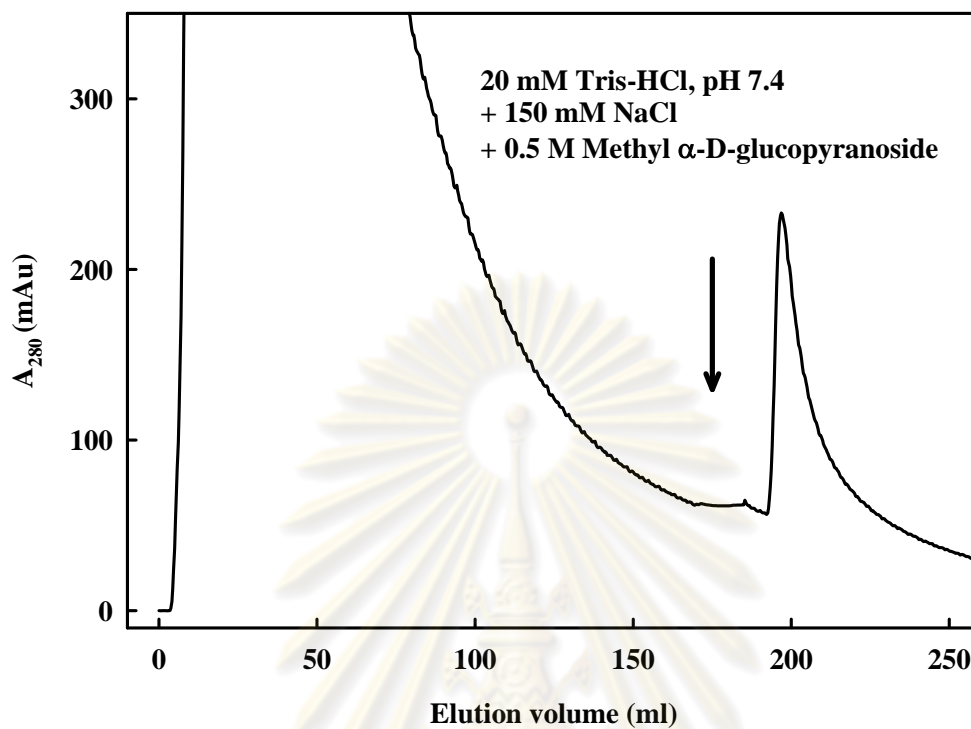
Figure 4.1 ConA Sepharose affinity chromatogram of the *K. parviflora* rhizome lectin, equilibrated and then washed with TBS. The lectin was then eluted with TBS containing 0.5 M Methyl α-D-glucopyranoside.
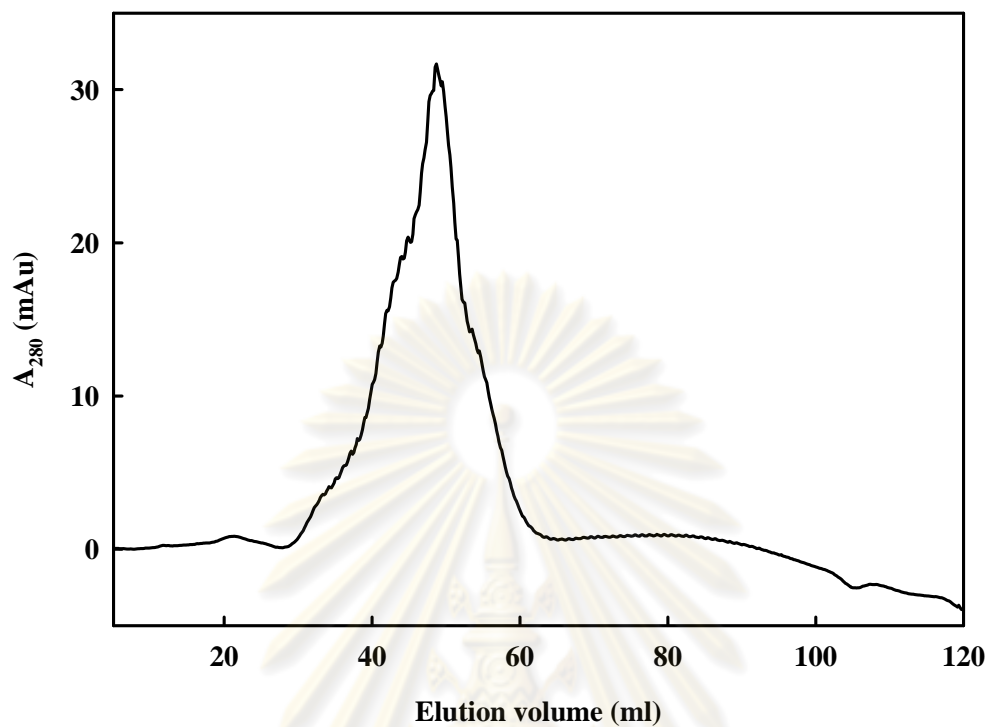
Figure 4.2 Elution profile of the purified *K. parviflora* lectin from a Sephacryl S-100 gel filtration chromatography column.

Table 4.1 Purification summary of the TBS-soluble *K. parviflora* rhizome lectin

Purification step	Total protein (mg)[a]	Total activity (HU)[b]	Specific activity (HU/mg)[c]	Yield (%)	Purification (fold)[d]
Crude extract	1958	25600	13.1	100	1
80% sat. (NH$_4$)$_2$SO$_4$ precipitation	1758	24000	13.7	94	1.04
Con A Sepharose (bound fraction)	19.5	19200	985	75	75.3
Gel Filtration (Sephacryl S-100)	8.72	10240	1174	40	89.8

[a] Crude protein extract from 334 g wet weight of rhizomes.
[b] Minimal concentration of protein able to cause visible agglutination of a 2 - 4% (v/v) suspension of rabbit erythrocytes.
[c] Specific activity is defined as the hemagglutination unit (HU) divided by the protein concentration (mg/ml) of the assay solution. Rabbit erythrocytes were used for the assay.
[d] Purification index was calculated as the ratio between the minimal concentration of the crude extract able to cause visible agglutination of the rabbit erythrocytes and that of the protein fraction obtained at each purification step.

Affinity chromatography procedures are widely used for lectin purification because of the relatively high specificity and good yields obtained, and the relative ease of binding (covalently linking) specific carbohydrates to insoluble naturally occurring or chemically modified resins, such as insoluble hog gestic musin, chitin, arabinogalactan, sephadex and agarose or sepharose (Lis and Sharon, 1986). For example, the mannose-glucose specific lectins from the seeds of the tepary bean (*Phaseolus acutifolius*) and mulberry, *Morus* sp. (Rosales: Moraceae), seeds were successfully purified by a single step ConA Sepharose based affinity chromatography column (Richard *et al*., 1990; Absar *et al*., 2005). Thus, fast and efficient procedures can be performed for lectin purification and, moreover, without inducing any modification after binding to carbohydrates (Lis and Sharon, 1980).

However, affinity chromatography has not always been applied successfully for the purification of some lectins, such as the isolation of *Dolichose biflorus* lectin with N-acetyl-galactosamine (NAG) immobilized to Sepharose and the lectin from ground elder (*Aegopodium podagraria*) rhizomes (Borrebaeck and Etzler, 1980; Peuman *et al*., 1985). In this study, the absence of any detectable hemagglutination

activity in the non-bound ConA sepharose elutant observed suggests no alternative such lectins are common in *K. parviflora* rhizomes, or at least in the TBS-soluble fraction, and thus the suitability of ConA sepharose affinity chromatography. Against this is that, affinity chromatography can be very expensive because of the requirement for specific materials with a high degree of purity which substantially increases the process costs. In addition the many pigments and oily compounds frequently found in crude plant extracts can impregnate the resin and damage the affinity column. From the total neutral carbohydrate analysis, this *K. parviflora* rhizome lectin was found to contain 14.7% (w/w) sugar, which is significantly higher than that reported before for the lectins from the Chinese evergreen chinkapin (5.8%), *Helianthus tuberosus* L. tubers (5.3%), *Helianthus tuberosus* L. tubers (5.3%) and *Arundu donex* (2.1%) (Suseelan *et al*., 2002; Kaur *et al*., 2005; Wong *et al*., 2008).

4.2 Molecular weight determination

Discontinuous SDS-PAGE in the presence of 2-merceptoethanol under reducing conditions, revealed a single strong band for the purified *K. parviflora* lectin corresponding to an apparent molecular weight of 41.7 kDa after coomassie blue R250 staining (Figure 4.3) In addition, since only a single band of the same apparent size was seen under non-reducing conditions (not shown) this suggests that the purified lectin could be a monomeric protein with hemagglutination activity alone. The apparent size of ~41.7 kDa is slightly larger than the previously published sizes of most other plant lectins, which range from 30 to 35 kDa (Franz *et al*., 1981; Van Damme *et al*., 1995; Kamemura *et al*., 1996), and is more in size agreement with that of the bacterial lectin from *E. coli* (Basu *et al*., 2004). Regardless, this higher apparent MW may reflect the greater degree of observed glycosylation.
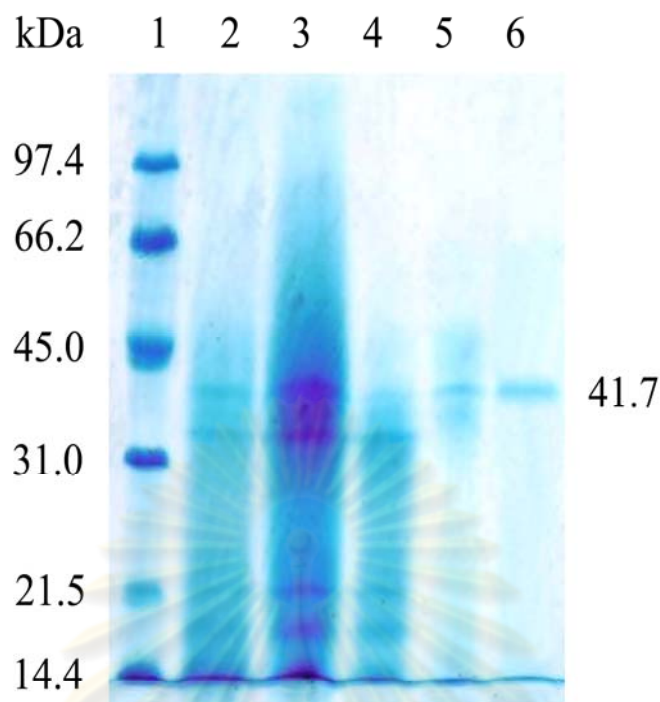
Figure 4.3 Reducing SDS-PAGE analysis of the *K. parviflora* rhizome lectin purification. Lane 1, molecular weight standards; Lane 2, the crude extract (homogenate); Lane 3, the 80% saturation ammonium sulphate precipitated and kept fraction; Lane 4, the discarded non-bound ConA sepharose fraction; Lane 5, the retained ConA sepharose-bound fraction; and Lane 6, the peak with hemagglutinating activity obtained from the Sephacryl S-100 gel filtration.

4.3 Assay for hemagglutinating activity

    K. parviflora lectin powerfully agglutinated rabbit erythrocytes with, in order of agglutination activity; rabbit >> sheep > rat = goose > mouse = guinea pig (Table 2). In addition it showed a weak but essentially non-specific agglutination of human erythrocytes where the A, B and AB groups showed an equal agglutination activity and group O was only marginally stronger but still weak, and on a par with that seen against mice and guinea pig erythrocytes (Table 4.2). The low hemagglutination of this lectin to mouse and guinea pig erythrocytes is similar to that seen with lectins from *Pisum sativum* and *Bauhinia monandra* seeds (Coelho and Silva, 2000; Sitohy *et al*., 2007), whilst the high hemagglutination levels seen with rabbit erythrocytes is broadly similar to that seen with the lectin from *Hevea brasiliensis* and *Canavalia cathartica* (Wittsuwannakul *et al*., 1998; Suseelan *et al*., 2009), but the combined species-activity profile of this lectin from *K. parviflora* is somewhat unique.

However, in terms of species specificity only, the lectin from *Trichosanthes anguina* exhibited hemagglutination activity against all four human group erythrocytes well as mouse, rabbit and goat erythrocytes (Shanavas *et al*., 1995). Examples of different species specificities include the seed lectins from *Longchocapus capassa* and *Galactia lindenii* which are specific for human group O as well as rabbit erythrocytes (Joubert *et al*., 1986; Almanza *et al*., 2009), but the two differ in activities and the latter also agglutinates rat erythrocytes. Also, mannose/glucose-specific lectin from Chinese evergreen chinkapin (*Castanopsis chinensis*) seeds which exhibited hemagglutinating activity against mouse and rabbit erythrocytes (Wong *et al*., 2008).

Table 4.2 Hemagglutinating activity of the purified *K. parviflora* rhizome lectin against human and other animal erythrocytes

Blood type	Total activity (HU)
Human A	640
Human B	640
Human AB	640
Human O	1,280
Rabbit	19,200
Mouse	1,200
Rat	4,800
Guinea pig	1,200
Geese	4,800
Sheep	9,600

The concentration of *K. parviflora* lectin used in these assays was 1 mg/ml and was serially 1:1 (v/v) diluted. Data shown are the mean $\pm$ 1 S.D. and are derived from three repeats

4.4 Effect of temperature on lectin hemagglutination activity and stability

The hemagglutinating activity of the purified *K. parviflora* rhizome lectin at 70 $^{\circ}$C and 80 $^{\circ}$C was found to decrease to 40% and 60%, respectively, of the

corresponding activity at room temperature, suggesting different conformational changes of the lectin under these conditions (Figure 4.4). This phenomenon, although not the exact temperature shift values, is quite similar to that seen for the *Phaseolus vulgaris* lectin (Biswas and Kayastha, 2002) which was found to be extremely thermostable at temperatures around 82 °C, to the lectin from *Astragalus mongholicus* which was still active at 65 °C (Yan *et al.*, 2005), and to the lectin from *Canavalia cathartica* which was active at 60 °C but not at 70 °C (Suseelan *et al.*, 2009).
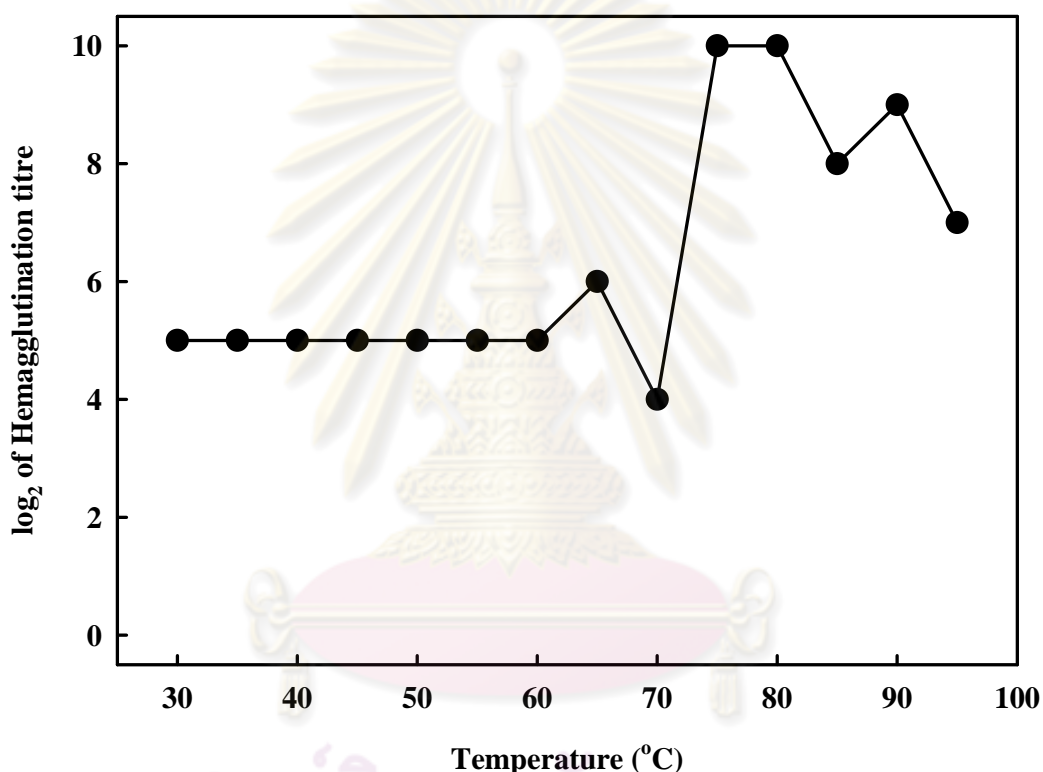
Figure 4.4 Effect of temperature on the agglutinating activity of the purified *K. parviflora* rhizome lectin towards a rabbit erythrocyte suspension in TBS. The data are shown as the mean $\pm$ 1 S.D. and are derived from three repeats. Full activity (100%) corresponds to a titer of 2^5.

Contrastingly, the hemagglutinating activity of the *Psophocarpus palustric* lectin rapidly declined when heated above 50 °C, being reduced to half at 60 °C and was completely lost at 70 °C (Adenike *et al.*, 2005), whilst the lectins from the rhizomes of *Smilax glabra, Aspidistra elatior* Blume and *Arundo donax* were more stable at temperatures up to 50 °C, although with very little hemagglutination activity

remaining above 80 - 85°C, and they showed no enhanced activity with increased temperature (Kaur *et al*., 2005; Xu *et al*., 2007). Together these data suggest that the hemagglutination activity depends on the native conformation of the protein (Ng and Yu, 2001). Indeed, the activity of the lectin is related to cations, just like the metal ions in Concanavalin A, which protect it from proteolytic and temperature degradation (Doyle and Keller, 1986).

The thermostability of the purified *K. parviflora* rhizome lectin, with respect to the hemagglutination activity, varied with both the incubation time and temperature (Figure 4.5). Pre-incubation at 70, 80 and, to a lesser extent, at 90 °C were found to increase the subsequent hemagglutination activity with pre-incubation times between 30 - 60 min and then stabilized at a higher (70 and 80 ^{o}C) or broadly the same (90 ^{o}C) final activity level than that with no preincubation. The markedly lower final activity seen at 90 ^{o}C than at the other two temperatures is presumably due to a lower enhanced activation level in the early preincubation period. In contrast, pre-incubation at 60 ^{o}C had a different pattern compared to other temperatures with the hemagglutination activity rapidly decreasing with increasing preincubation time until fully inactive after 90 min. This result potentially implies that the conformational structure adopted by the lectin structure at 60 ^{o}C is not suitable for hemagglutination.

Figure 4.5 Thermostability of the same purified lectin towards a rabbit erythrocyte suspension in TBS at: (○) 60 ^{o}C, (●) 70 ^{o}C, (■) 80 ^{o}C and (▲) 90 ^{o}C. The data are shown as the mean $\pm$ 1 S.D. and are derived from three repeats. Full activity (100%) corresponds to a titer of 2^5.
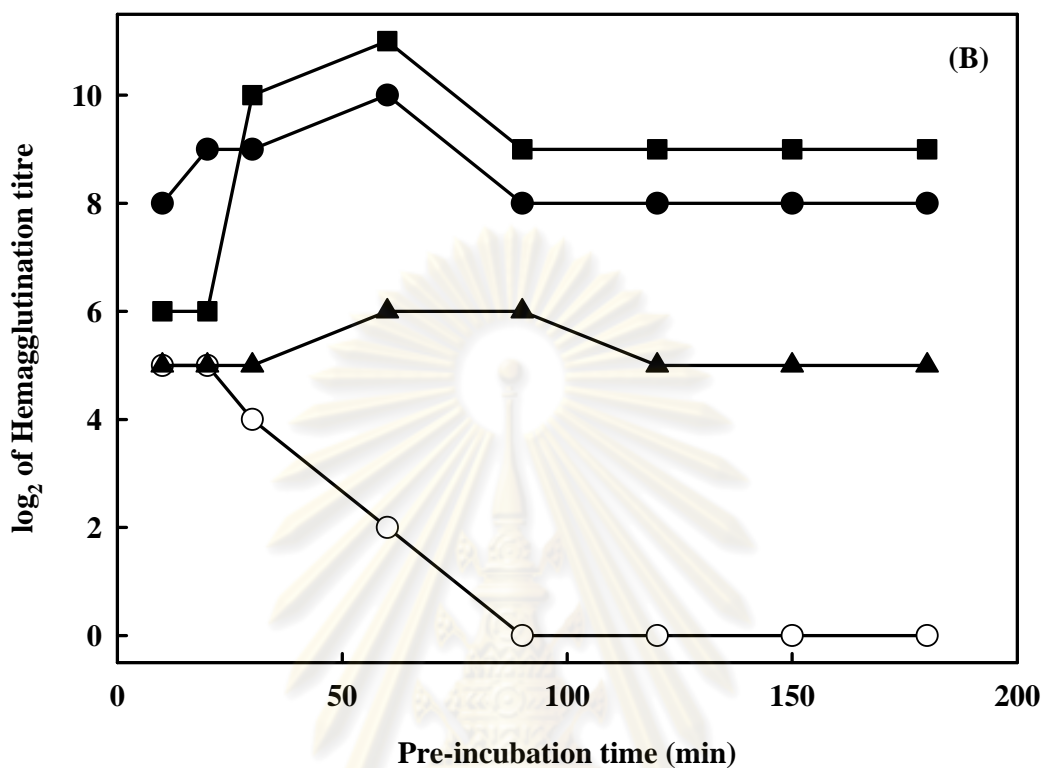
The Gibb's free energy of thermostability at all incubation temperatures and times was calculated, and found to vary significantly with both positive and negative values. For example, the ΔG at 60 °C and 70 °C preincubation for 30 minutes were found to be 58.29 and -60.56 cal/ mol, respectively. Since $\Delta G = \Delta H - \Delta(ST)$, at a constant temperature ΔT is zero and ΔG depends on the ΔH term. If ΔG is positive, energy is absorbed in the non-spontaneous process, whereas if ΔG is negative energy is evolved in the spontaneous process. Considering that the high hemagglutination activity seen at 70 °C for 20 minutes corresponds to a ΔG of 59.82 cal/ mol. The stability of the *K. parviflora* rhizome lectin is maximal at the temperature at which the entropies of the native and denatured state are equal. Because many lectins exert anti-

nutritional effects on mammals including humans (Vasconcelos and Oliveira, 2004), the determination of the free energy of activation of the lectin denaturation process is an important physicochemical parameter, especially considering the increasing applications of lectins in agriculture, medicine and related areas. This information is also relevant from the nutritional point of view, taking into account the potential use in human and animal nutrition.

4.5 Effect of pH on lectin hemagglutination activity

An aliquot of each purified lectin in distilled water was lyophilized and dissolved in the different buffer-pH combinations to assay its hemagglutination activity across the pH 2 - 12 range, inclusive, in integer increments. After a two-fold dilution of each buffer with TBS the pH was reverted to pH 7.4 and the hemagglutination activity test was then performed with rabbit erythrocytes, since they were found to be more tolerant, in terms of hemagglutination, to different kinds of buffer. That said, however, a clear difference in the hemagglutination activity was observed between the sodium acetate and potassium phosphate buffers at pH 6, and also between the Tris-HCl and the glycine-NaOH buffers at pH 10 (Figure 4). Whether these are an artifact of the nature of the buffer or they affect the interaction of the lectin itself is unclear. Nevertheless, that aside for the present, the purified *K. parviflora* rhizome lectin was found to be optimal, in terms of hemagglutination activity, at pH 7 but relatively stable at pH 6, and stable at 50% activity within the pH range of 3 - 6 and 8 - 10 inclusive. Thus, at more acidic (<pH 3) and alkaline (>pH 10) levels the hemagglutination activity was significantly reduced (Figure 4.6).

This pH optima and loss at alkali and acid conditions is broadly similar to that seen with the lectin from *Salvia bogotensis* seeds where the maximum pH stability was noted at pH 7.0 and 8.0 and declined to zero at below pH 2.0 and above pH 12.5 (Vega and Perez, 2006), and the 50% loss of hemagglutination activity at pH 8.5 for the *Helianthus tuberosus* L. tuber lectin (Suseelan *et al*., 2002). In addition, the lectins from *Parkia javanica* (Utarabhand and Akkayanont, 1995), and *Clitoria ternatea* (Naeem et al., 2007) exhibited a pH stability between 7 - 10 and 7 - 7.5 respectively, whilst the Chinese chestnut, *Castanea mollisima*, lectin was unstable in the presence of acid and alkali (Ng *et al*., 2002). However, other lectins have been reported to be stable over a broader pH range, such as those from *Koelreuteria paniculata* seeds,

Talisia esculenta and *Sphenostyles stenocarpa* (Machuka *et al*., 1999; Freire *et al*., 2002; Macedo *et al*., 2003).
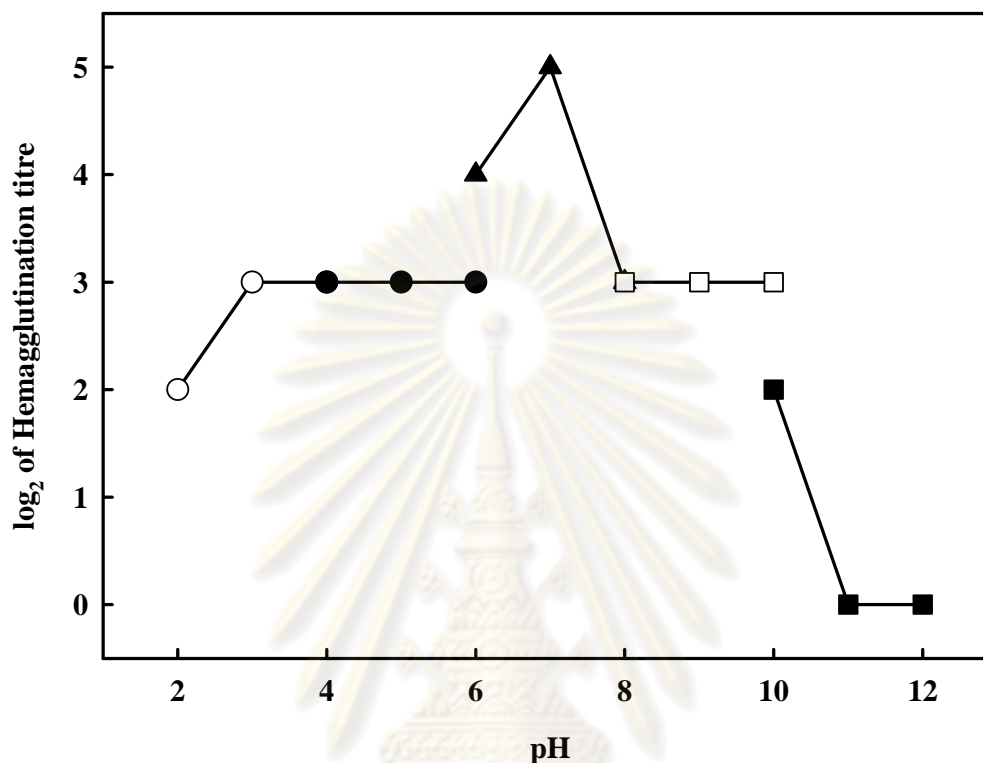
Figure 4.6 The effect of pH on the hemagglutinating activity of *K. parviflora* rhizome lectin towards rabbit erythrocytes. The data are shown as the mean $\pm$ 1 S.D. and are derived from three repeats. Full activity (100%) corresponds to a titer of 2^5. The following buffer systems were used: ($\circ$) 20 mM glycine-HCl (pH 2.0 - 4.0), ($\bullet$) 20 mM sodium acetate (pH 4.0 - 6.0), ($\blacktriangle$) 20 mM potassium phosphate (pH 6.0 - 8.0), ($\square$) 20 mM Tris-HCl (pH 8.0 - 10.0) and ($\blacksquare$) 20 mM glycine-NaOH (pH 10.0 - 12.0).

4.6 Effect of metal ions

The hemagglutination activity of the purified *K. parviflora* lectin was supported, in order of effectivity, by $Mn^{2+} > Ca^{2+} = Mg^{2+}$, but in contrast, Mg^{2+}, Fe^{3+}, Hg^{2+}, Co^{2+} and EDTA did not support hemagglutinating activity (Table 4.3). Similar results have been observed for the *Dioclea altissima* lectin (Moreira *et al*., 1997). Indeed, the great majority of lectins studied so far contain metal ions, normally Ca^{2+}, Mg^{2+} or and Mn^{2+}, and often require metal ions for maintenance of conformation and their ability to bind carbohydrate (Goldstein and Poretz, 1986; Sharon and Lis, 1990).

In contrast, the lectin from the rhizomes of *Smilax glabra* was not affected by the monovalent cations (Na^+, K^+ and NH^{4+}), the divalent cations (Ca^{2+}, Mg^{2+}, Mn^{2+} and Cu^{2+}) and the trivalent cation Fe^{3+} (Ng and Yu, 2001), whilst the presence of 0.05 M EDTA did not effect the agglutinating activity of the lectin from *Erythrina cristagalli*.

Table 4.3 The effect of divalent metal cations and EDTA on the hemagglutination activity of the *K. parviflora* rhizome lectin.

Metal ion	Concentration (mM)			
	12.5	25	50	100
Ca^{2+}	-	+	+	+
Mn^{2+}	+	+	+	+
Mg^{2+}	-	+	+	+
Fe^{3+}	-	-	-	-
Hg^{2+}	-	-	-	-
Co^{2+}	-	-	-	-
EDTA	-	-	-	-

+ hemagglutinating activity
- no hemagglutinating activity

4.7 Internal amino acid sequence of lectin by LC/MS/MS

The internal sequence analysis of the purified lectin from *K. parviflora* rhizomes was obtained by digestion with trypsin and sequence analysis with LC/MS/MS and this was found to be GPIQL SYNYN YGPAG K (m/z of 1742.91) (Figure 4.7). Comparisons were then made to all protein sequences in the SwissProt database using BLASTp searches. A high degree of internal amino acid sequence identity between the lectin from *K. parviflora* rhizomes and other lectins suggested that this protein could be a member of lectin/endochitnase1 family (Lerner and Raikhel, 1992), as shown in Figure 4.8.

Figure 4.7 Amino acid sequence from the tryptic fragments of the purified *K. parviflora* lectin. Comparisons are made with other lectins from the lectin family that showed the highest sequence homology in BLASTp searches of the NCBI and SwissProt databases. Shaded regions represent regions of identity.
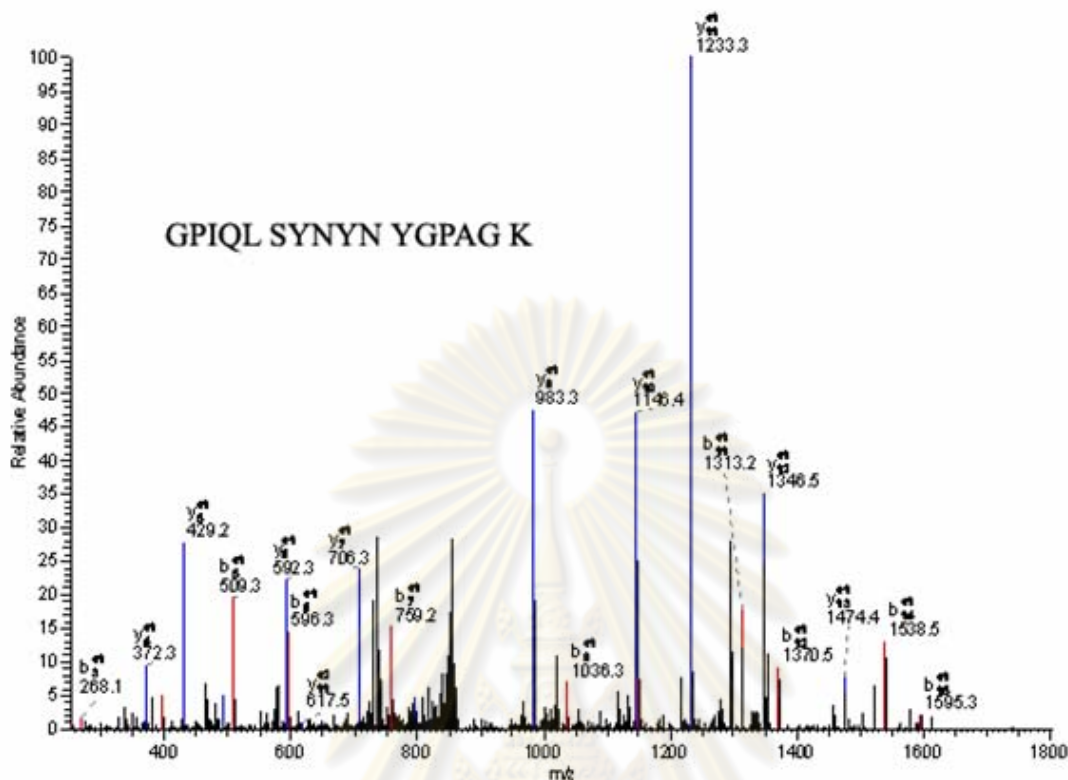
Figure 4.8 LC/MS/MS spectra of the tryptic digest of the purified lectin used to derive the data in Figure 4.7 above.

4.8 Assay of antifungal activity

A clear antifungal activity by the *K. parviflora* lectin against the three tested species of plant pathogenic fungi, *E. turcicum*, *F. oxysporum* and *C. cassicola*, was observed, with this being strongest against *C. cassicola* followed by *E. turcicum* and *F. oxysporum*, respectively (Figure 4.9). Although proteins from ginger rhizomes have been reported to exhibit antifungal activity towards a variety of fungi, including *B. cinerea, F. oxysporum, Mycosphaerella arachidicola* and *Physalospora piricola* (Wang and Ng, 2005), this is the first report of a lectin with such activity in the Zingiberaceae family to our knowledge. Although many studies of plant lectins have assumed that they are implicated in host defense mechanisms as antifungal proteins, to date, however, only a small number of plant lectins have been reported to actually have antifungal activity, including those from potato tubers, *Amaranthus caudatus* seeds, stinging nettle rhizomes, wheat germ and *Phaseolus vulgaris* seeds (Broekaert

and Peumans, 1986; Gozia *et al.*, 1993; Verheyden *et al.*, 1995; Yu *et al.*, 2001). Moreover, some selectivity is noted. For example, the root lectin from *Astragalus mongholicus* (AMML) revealed antifungal activity against *B. cincerea*, *F. oxysporum*, *Colletorichum* sp., and *Drechslera turcia* but not against *Rhizoctonia solani* and *M. arachidicola* (Yan *et al.*, 2005). Similarly, the lectin from *T. esculenta* seeds and monomeric mannose/glucose-binding lectin from red cluster pepper (*Capsicum frutescens*) inhibited the growth of *F. oxysporum*, *C. lindemuthianum* and *S. cerevisiae* (Freire *et al.*, 2002), or *Aspergillus flavus* and *Fusarium moniliforme* spores and hyphal growth (Ngai and Ng, 2009), respectively. Moreover, *in vitro* studies have demonstrated that two novel chitin-binding lectins from the seeds of Artocarpus integrifolia inhibited the growth of *F. moniliforme* and *S. cerevisae* (Trindale *et al.*, 2006).
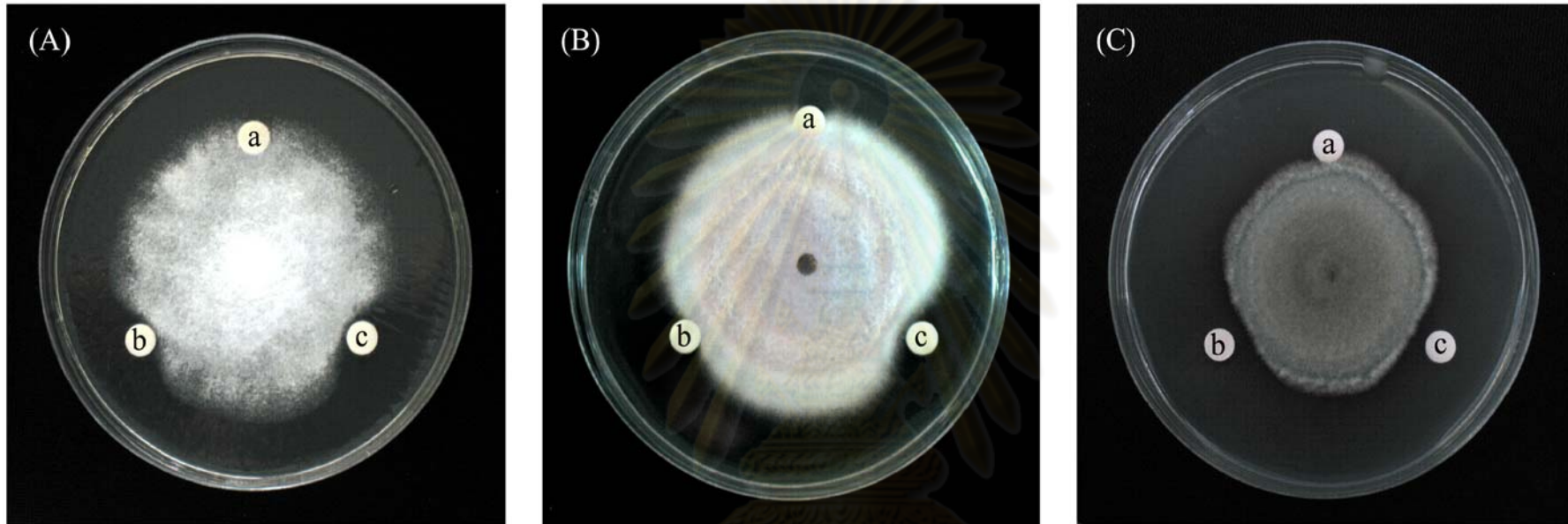
Figure 4.9 The antifungal (colony growth inhibition) effect of the *K. parviflora* rhizome lectin towards (A) *E. turicicum,* (B) *F. oxysporum* and (C) *C. cassiicola*, showing the (a) negative control (10 μl of 20 mM Tris-HCl buffer pH 7.4), (b) 18 μg/ 0.3 cm^2 disc and (c) 36 μg/ 0.3 cm^2 disc of *K. parviflora* rhizome lectin.

4.9 Assay of bacterial activity

K. parviflora lectin exhibited moderate antimicrobial activity against the gram positive bacteria *B. subtilis* (MIC of 0.184 mg/ml) and weaker activity against the gram negative and positive *P. aeruginosa* and *S. aureus*, respectively, both with a MIC value of 1.270 mg/ml. However, no inhibitory activity was detected against *E. coli* (gram negative) or the yeast *C. albican*s. Whether this truly demonstrates a lack of specificity for gram-positive bacteria over gram-negative bacteria remains to be confirmed by evaluation of more diverse species within each category. However, the binding of lectins to muramic acid and N-acetylmuramic acid, carbohydrates present mainly in gram-positive bacterial cell walls, has been reported (Ayouba *et al*., 1991), and given that they are at low levels in *E. coli*, it may be informative to screen lectin anti-bacterial activity against expression levels of these two membrane components. In addition, these data suggest that lectins could potentially play a role in plant defense, not only against phytopathogenic invertebrates, herbivores or fungi, but also against bacteria. The carbohydrate-binding site probably plays a key role in this activity, being responsible for the recognition of bacteria. Almost all microorganisms express surface-exposed carbohydrates. These carbohydrates may be covalently bound, as in glycosylated teichoic acids to peptidoglycan, or non-covalently bound, as in capsular polysaccharides (Hirmo *et al*., 1997; Caldeon *et al*., 1997). Every surface-exposed carbohydrate is a potential lectin-reactive site. The ability of lectins to form complexes with microbial glycoconjugates has made them useful tools as probes and sorbents for whole cells, mutants, and numerous cellular constituents and metabolites.

4. 10 Alpha-glucosidase inhibition activity

Alpha-glucosidase inhibitors have been used for preventing the digestion of carbohydrates, with the aim of developing them as drugs for the dietary control of diabetes mellitus type 2 (Holman, 1998), via reduction of the impact of dietary complex carbohydrates on blood sugar levels. In addition, anti-HIV research has in part focused on compounds that interfere with viral binding and fusion including α-glucosidase activity since the binding of the HIV viral surface glycoprotein gp120 to the cellular viral receptor CD4 is dependent on the state of glycosylation of gp120, where both hyper- and hypo-glycosylated forms are not able to bind to the cell receptor. Thus, compounds that interfere with accurate carbohydrate processing of

this viral glycoprotein may prevent viral binding to cellular receptors and hence may be useful anti-viral agents (De Clercq, 1995; Asano, 2003; Jacob, 1995). However, there are only a few proteins in different plant sources that are reported to have an α-glucosidase inhibitory activity. Recently, two proteins, Dolichin, isolated from field beans (*Dolichos lablab*) (Ye *et al*., 2000), and Unguilin, isolated from seeds of the black-eyed pea *(Vigna unguiculata)* (Ye and Ng, 2001), were reported to be able to inhibit human immunodeficiency virus (HIV) reverse transcriptase, as well as α- and β-glucosidases which are glycohydrolases implicated in HIV infection. In present study, the half Inhibition Concentration (IC_{50}) of *K. parviflora* lectin was found to be about 1.89 μg/ml of protein.

CHAPTER V

CONCLUSION

The TBS-soluble lectin extracted from *Kaempferia paviflora* rhizomes was successfully purified in two steps by Concanavalin A affinity chromatography and Sephacryl S-100 gel filtration on Sephacryl S-100. The purification of the protein from the crude extract to the bound lectin on gel filtration led to an increasedincrements in the specific activity up to 1,174 HU/mg protein, corresponding to a 40% yield. Reducing SDS-PAGE analysis yielded an estimated molecular weight for this lectin of about 41.7 kDa. The lectin showed non-specific weak hemagglutinating activity on human (A, B, AB and O) erythrocytes, as well as stronger activity on rabbit >> sheep > goose = rat> mouse = guinea pig erythrocytes, respectively. Suitable cofactors for the lectin's hemagglutination activity were Mn^{2+}> $Mg^{2+} = Ca^{2+}$. The optimum pH and temperature of lectin were between 6 - 8 and 75 - 85 °C, respectively, suggesting that the *K. paviflora* lectin is a thermally stabile lectin. The *K. parviflora* rhizome lectin exhibited moderate antimicrobial activity against some gram negative and gram positive bacteria, with a moderate activity against *B. subtilis* (MIC = 0.184 mg/ml), and a weaker activity against S. aureus and *P. aeruginosa* (MIC = 1.270 mg/ml), but no detectable activity against either *E. coli* or the yeast, *C. albicans*. The α-glucosidase inhibition activity was weak, with an IC_{50} of 1.89 μg/ml, and is, therefore, not a likely prime candidate alone for HIV, diabetes type 2 and other related α-glucosidase inhibitory based therapeutic applications.

REFERENCE

Ahmad, N., Srinivas, V.R., Reddy, B.H. and Surolia, A. 2009. Thermodynamic characterization of the conformational stability of the homodimeric protein, Pea Lectin. Biochemistry 37 : 16765-16772.

Ahmad, S., Khan, R.H. and Ahman, A. 1999. Physicochemical characterization of *Cajanus cajan* lectin: effect of pH and metal ions on lectin carbohydrate interaction. Biochimica et Biophysica Acta (BBA) - General Subjects 1427 : 378-384.

Ahmed, H. and Chatterjee, B.P. 1989. Further characterization and immunochemical studies on the carbohydrate specificity of jackfruit (*Artocarpus integrifolia*) lectin. Journal of Biological Chemistry 264(16) : 9365-9372.

Almanza, M., Vega, N. and Perez, G. 2004. Isolating and characterising a lectin from *Galactia lindenii* seeds that recognises blood group H determinants. Archives of Biochemistry and Biophysics 429 : 180-190.

Apenten, R.K.O., Khokhar, S. and Galani, D. 2002. Stability parameters for β-lactoglobulin thermal dissociation and unfolding in phosphate buffer at pH 7.0. Food Hydrocolloids 16(2) : 95-103.

Bell, H A., Fitches, E. C., Down, R. E., Marris, G.C., Edword, J.C., Gatehouse, J.A. and Gatehouse, A.M.R. 1998. The effect of snowdrop lectin (GNA) delivered via artificial diet and transgenic plants on *Eulophus pennicornis* (Hymenoptera: Eulophidae), a parasitoid of the tomato moth *Lacanobia oleracea* (Lepidoptera: Noctuidae). Journal of Insect Physiology 45(11) : 983-991.

Bhandari, M.R., Jong-Anurukkun, N., Hong, G. and Kawabata, J. 2008. α-Glucosidase and α-amylase inhibitory activities of Nepalese medicinal herb Pakhanbhed (Bergenia ciliata, Haw.). Food Chemistry 106(1) : 247-252.

Biswas, S. and Kayastha, A.M. 2002. Thermal stability of *Phaseolus vulgaris* leucoagglutinin: a differential scanning calorimetry study. Journal of Biochemistry and Molecular Biology 35(5) : 472-475.

Brownlee, M. and Cerami, A. 1979. A glucose-controlled insulin-delivery system: semisynthetic insulin bound to lectin . Science 206(4423) : 1190-1191.

Chandarana, H., Baluja, S. and Chanda, S.V. 2005. Comparison of antibacterial activities of selected species of Zingiberaceae family and some synthetic compounds. Turkish Journal of Biology 29 : 83-97.

Chen, I.N., Chang, C.C., Ng, C.C., Wang, C.Y., Shyu, Y.T. and Chang, T.L. 2008. Antioxidant and antimicrobial activity of Zingiberaceae plants in Taiwan. Plant Foods for Human Nutrition 63(1) : 15-20.

Du, Z.Y., Liu, R.R., Shao, W.Y., Mao, X.P., Ma, L., Gu, L.Q., Huang, Z.S. and Chan, A.S.C. 2006. α-Glucosidase inhibition of natural curcuminoids and curcumin analogs. European Journal of Medicinal Chemistry 41(2) : 213-218.

Emadelin, H.E., Mulay, R., Faca, V., Johnward, R., Joel, G,L., Roque-Barriera, M.C., Sabharwal, S. and Bhide, S. V. 2002. Purification, some properties of a D-galactose-binding leaf lectin from *Erythrina indica* and further characterization of seed lectin. Biochimie 84 : 1035-1043.

Fiedler, K. and Simons, K. 1996. Characterization of VIP36, an animal lectin homologous to leguminous lectins. Journal of Cell Science 109(1) : 271-276.

Firon, N., Ofek, I. and Sharon, N. 1984. Carbohydrate-binding sites of the mannose-specific fimbrial lectins of enterobacteria. Infection and Immunity 43(3) : 1088-1090.

Gade, W., Jack, M.A., Dahl, J.B., Schnidt, E.L. and Wold, F. 1981. The isolation and characterization of a root lectin from soybean (*Glycine max* L), cultivar Chippewa). Journal of Biological Chemistry 256(24) : 12905-12910.

Glabe, C.G., Harty, P.K. and Rosen, S.D. 1983. Preparation and properties of fluorescent polysaccharides . Analytical Biochemistry 130(2) : 287-294.

Hammaar, L., Hissch, I., Machado, A., De Mareuil, J., Baillon, J. and Chermann, J.C. 2009. Lectin effects on HIV-1 infectivity. Annals of the New York Academy of Sciences 724 : 166-169.

Hirabayashi, J., Hashidate, T., Nishi, N., Nagamura, T., Hirashima, M., Urashima, T., Oka, T., Futai, M., Muller, W. E. G., Yagi, F. and Kasai, K. 2002. How galectins have evolved oligosaccharide specificity?. Biochim Biophys Acta 1572 : 263-273.

Hori, K., Miyazawa, K. and Ito, K. 1990. Some common properties of lectins from marine algae . Hydrobiologia 204-205 : 561-566.

Inbar, J. and Chet, I. 1992. Biomimics of fungal cell-cell recognition by use of lectin-coated nylon fibers. The Journal of Bacteriology 174(3) : 1055-1059.

Inbar, J. and Chet, I. 1994. A newly isolated lectin from the Plant pathogenic fungus *Sclerotium roltsii*: purification, characterization and role in mycoparasitism. Microbiology 140(3) : 651-657.

Iobst, S.T. and Drickamer, K. 1994. Binding of sugar ligands to Ca^{2+}-dependent animal lectins. II. Generation of high-affinity galactose binding by site-directed mutagenesis. Journal of Biological Chemistry 269(22) : 15512-15519.

Jantan, I., Rawsh, S. M., Sirat, H.M., Jamil, S., Moad, Y.Y.H., Jalil, J. and Jamal, J.A. 2008. Inhibitory effect of compounds from Zingiberaceae species on human platelet aggregation. Phytomedicine 15(4) : 306-309.

Joubert, F.J., Sharon, N. and Merrefield, E.H. 1986. Purification and properties of a lectin from *lonchocarpus capassa* (apple-leaf) seed. Phytochemistry 25(2) : 323-327.

Keitel, U., Simon, O., Borriss, R. and Heinemann, U. 1993. Molecular and activesite structure of a Bacillus 1,3-1,4-β-glucanase. Proceedings of the National Academy of Sciences USA 90 : 5287-5291.

Kim, K.Y., Nam, K.A., Kurihara, H. and Kim, S.M. 2008. Potent alpha-glucosidase inhibitors purified from the red alga *Grateloupia elliptica*. Phytochemistry 69(16) : 2820-2825.

Kim, Y.M., Wang, M.H. and Rhee, H.I. 2004. A novel α-glucosidase inhibitor from pine bark. Carbohydrate Research 339(3) : 715-717.

Kolatkar, A. R. and Weiss, W. I. 1996. Structural basis of galactose recognition by C-type animal lectins. Journal of Biological Chemistry 271 : 6679-6685.

Konozy E.H.E., Bernardes E.S., Rosa, C., Faca, V., Greene L.J. and John W.R. 2003. Isolation, purification, and physicochemical characterization of a D-galactose-binding lectin from seeds of *Erythrina speciosa*. Archives of Biochemistry and Biophysics 410 : 222-229.

Koo, B.S., Lee, W.C., Chung, K.H., Ko, J.H. and Kim, C.H. 2004. A water extract of *Curcuma longa* L. (Zingiberaceae) rescues PC12 cell death caused by pyrogallol or hypoxia/reoxygenation and attenuates hydrogen peroxide induced injury in PC12 cells. Life Sciences 75(19) : 2363-2375.

Kuhn, P., Tarentino, A.L., Plummer, T.H. and Roey Van, P. 1994. Crystal structure of peptide-N(4)-(N-acetyl-β-D-glucosaminyl) asparagine amidase at 2.2 A° resolution. Biochemistry 33 : 11699-11706.

Kumar, A. and Singh, A. 2008. Possible nitric oxide modulation in protective effect of (*Curcuma longa*, Zingiberaceae) against sleep deprivation-induced behavioral alterations and oxidative damage in mice. Phytomedicine 15(8) : 577-586.

Kunjeshwori Devi, S., Inaotombi Devi, L. and Rupachanda Singh, L. 2009. Purification and Characterization of a New Dimeric Mannose/Glucose-Binding Isolectin from *Vicia tetrasperma* (L.) Schreber. Preparative Biochemistry & Biotechnology 39 : 57-71.

Lantz, R.C., Chen, G.J., Solyom, A.M., Jolad, S.D. and Timmermann, B.N. 2005. The effect of turmeric extracts on inflammatory mediator production. Phytomedicine 12(6-7) : 445-452.

Liu, C., Zhao, X., Xu, X., Li, L., Liu, Y., Zhong, SH. and Bao, J. 2007. Hemagglutinating activity and conformation of a lactose-binding lectin from mushroom *Agrocybe cylindracea*. International Journal of Biological Macromolecules 42 : 138-144.

Mccrary, B.S., Edmondson, S.P., and Shriver, J.W. 1996. Hyperthermophile Protein folding thermodynamics: Differential scanning calorimetry and chemical denaturation of Sac7d. Journal of Molecular Biology 264(4) : 784-805.

Mehul, B., Bawumia, S., Martin, S.R. and Hughes, R.C. 1994. Structure of baby hamster kidney carbohydrate-binding protein CBP30, an S-type animal lectin. Journal of Biological Chemistry 269(27) : 18250-18258.

Mirelman, D., Altmann, G. and Eshdat, Y. 1980. Screening of bacterial isolates for mannose-specific lectin activity by agglutination of yeasts. Journal of Clinical Microbiology 11(4), 328-331.

Montfort, W., Villafranca, J.E., Monzingo, A.F., Ernst, S. R., Katzin, B., Rutenber, E., Xuong, N. H., Hamlin, R. and Robertus, J. D. 1987. The three-dimensional structure of ricin at 2.8A°. Journal of Biological Chemistry 262 : 5398-5403.

Mozo-Villiarias, A. and Querol, E. 2006. Theoretical analysis and computational predictions of protein thermostability. Current Bioinformatics 1 : 25-32.

Ng, T.B., Yu, Y.L. and Chu, K.T. 2002. Isolation of a novel legumin-like lectin with potent hemagglutinating activity from seeds of the Chinese chestnut *Castanea mollisima*. Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology 133(3) : 453-460.

Ngai P.H.K. & Ng, T.B. 2009. A lectin with antifungal and mitogenic activities from red cluster pepper (*Capsicum frutescens)* seeds. Applied Microbiology and Biotechnology 74 : 366-371.

Ngai, P.H.K. and Ng, T.B. 2003. Lentin, a novel and potent antifungal protein from shitake mushroom with inhibitory effects on activity of human immunodeficiency virus-1 reverse transcriptase and proliferation of leukemia cells. Life Sciences 73(26) : 3363-3374.

Nishioka, T., Kawabata, J. and Aoyama, Y. 1998. Baicalein, an α-glucosidase inhibitor from *Scutellaria baicalensis*. Journal of Natural Products 61(11) : 1413-1415.

Oda, Y., Senaha, T., Matsuno, Y., Nakajima, K., Naka, R., Kinoshima, M., Honda, E., Furuta, I. and Kakehi, K. 2003. A new fungal lectin recognizing α (1-6)-linked fucose in the N-glycan. Journal of Biological Chemistry 278(34) : 32439-32447.

Oleh, I.O., I.K. Har, D.P., Bach, M. and Lemie, R.U. 1985. Molecular recognition. 111. The binding of the H-type 2 human blood group determinant by the lectin I of *Ulex europaeus*. Canadian Journal of Chemistry 63 : 2653-2658.

Pando, S.C., Macedo, M.L., Freire, M.G., Toyama, M.H., Novello, J.C. and Marangoni, S. 2002. Biochemical characterization of a lectin from *Delonix regia* seeds. Journal of *Protein Chemisty*. 21(4) : 279-285.

Patanasethanont, D., Nagai, J., Yumoto, R., Muragami, T., Sutthanut, K., Sripanidkulchai, B. O., Yenjai, C. and Takano, M. 2007. Effects of *Kaempferia parviflora* extracts and their flavone constituents on P-glycoprotein function. Journal of Pharmaceutical Sciences 96(1) : 223-233.

Raikhel, N.V., Lee, H.I. and Broekaer, W.F. 1993. Structure and function of chitin-binding proteins. Annual Review of Plant Physiology and Plant Molecular Biology 44 : 591-615.

Reisner, Y., Itzicovitch, L., Meshorer, A. and Sharon, N. 1978. Hemopoietic stem cell transplantation using mouse bone marrow and spleen cells fractionated by lectins. Proceedings of the National Academy of Sciences of the United States of America 75(6) : 2933-2936.

Riera, A.S., Daud, A., Gallo, A., Genti, S., Aybar, M. and Sanshez, S. 2003. Antibacterial activity of lactose-binding lectins from Bufo arenarum skin. Biocell 27(1) : 37-46.

Roberto , A.S., Francis, S.G., Thiago, H.N., Nataly, D.L.S., Carla, M.L.M., Norma, B. G., Luana , C.B.B. C., Patricia, M.G.P. and Lothar, W.B. 2009. Antibacterial and antifungal activities of *Myracrodruon urundeuva* heartwood. Wood Science and Technology 43 : 85-95.

Rujjanawate, C., Kanjanapothi, D., Amornlerdpison, D. and Pojanagaroon, S. 2005. Anti-gastric ulcer effect of *Kaempferia parviflora*. Journal of Ethnopharmacology 102(1) : 120-122.

Sampaio, A.H., Rogers, D.J. and Barwell, C.J. 1998. A galactose-specific lectin from the red marine algae *Ptilota filicina*. Phytochemistry 18(5) : 765-769.

Santos, A.F.S., Luz, L.A., Argolo, A.C.C., Teixeira J.A., Paiva, P.M.C. and Coelho L. C.B.B. 2009. Isolation of a seed coagulant *Moringa oleifera* lectin. Process Biochemistry 44 : 504-508.

Sharon, N. and Lis, H. 1990. Legume lectins-a large family of homologous proteins. Journal of the Federation of American Societies for Experimental Biology 4 : 3198-3208.

Singh, J., Singh, J. and Kamboj, S.S. 2004. A novel mitogenic and antiproliferative lectin from a wild cobra lily, *Arisaema flavum* . Biochemical and Biophysical Research Communications 314(4) : 1057-1065.

Sinha, S., Mitra, N., Kumar, G., Bajaj, K. and Surolia, A. 2005. Unfolding studies on soybean agglutinin and concanavalin a tetramers: A Comparative account. Biophysical Journal 88(2) : 1300-1310.

Sookkongwaree, K., Geitmann, M., Roengsumran, S., Petsom, A. and Danielson, U. H. 2006. Inhibition of viral proteases by Zingiberaceae extracts and flavones isolated from *Kaempferia parviflora*. Pharmazie 61(8) : 717-721.

Suseelan, K. N., Bhagwath, A., Pandey, R. and Gopalakrishna, T. 2007. Characterization of Con C, a lectin from *Canavalia cathartica* Thouars seeds. Food Chemistry 104 : 528-535.

Suzuki, T., Takagi, T., Furukohri, T., Kawamura, K. and Nakauchi, M. 1990. A calcium-dependent galactose-binding lectin from the tunicate *Polyandrocarpa misakiensis*. Isolation, characterization, and amino acid sequence. Journal of Biological Chemistry 265(3) : 1274-1281.

Sweet, R.M., Wright, H.T., Janin, J. and Chothia, C. 1974. Crystal structure of the complex of porcine trypsin with soybean trypsin inhibitor (Kunitz) at 2.6-A° resolution. Biochemistry 13 : 4212-4228.

Tewtrakul, S., Subhadhirasakul, S. and Kummee, S. 2008. Anti-allergic activity of compounds from *Kaempferia parviflora*. Journal of Ethnopharmacology 116(1) : 191-193.

Van Damme, E.G., Goldstein, U., Vercamman, G., Vuylsteke, J. and Peumans, W.J. 2009. Lectins of members of the Amaryllidaceae are encoded by multigene families which show extensive homology. Plant Physiology 86 : 245-252.

Van Damme, E.G., Smeets, K., Torrekens, S., Van Leuven, F. and Peumans, W.J. 2009. Characterization and molecular cloning of mannose-binding lectins of the *Orchidaceae* species *Listera ovate, Epipactis belleborine* and *Cymbidium hybrid.* European Journal of Biochemistry 93 : 769-777.

Vichitphan, S., Vichiphan, K. and Sirikhansaeng, P. 2007. Flavonoid content and antioxidant activity of Kracahi-dun (*Kaempferia parviflora*) wine. KMITL Science and Technology Journal 7 : 97-105.

Voravuthikunchai, S.P., Limsuwan, S., Supapol, O. and Subhadhiruksakul, S. 2006. Antibacterial activity of extracts from family Zingiberaceae against foodborne pathogens. Journal of Food Safety 26 : 325-334.

Wang, H. and Ng, T.B. 2005. An antifungal protein from ginger rhizomes. Biochenical and Biophysical Research Communication 336(1) : 100-104.

Wang, H., Ng, T.B. and Ooi, V.E.C. 1998. Lectins from mushrooms. Cambridge Journal 102(8) : 897-906.

Wang, H.X. and Ng, T.B. 2009. Purification of castamollin, a novel antifungal protein from Chinese chestnuts. Protein Expression and Purification 32 : 44-51.

Wang, H.X., Ng, T.B.L. W.K., Oou, V.E.C. and Chang, S.T. 1995. Isolation and characterization of two distinct lectins with antiproliferative activity from the cultured mycelium of the edible mushroom *Tricholoma mongolicum.* International Journal of Peptide and Protein Research 46(6) : 508-513.

Wang, X.C., Bauw, G., Van Damme, E.J.M., Peumans, W.J., Chen, Z.L., Van Montagu, M., Angenon, G. and Dillen, W. 2001. Gastrodianin-like mannose-binding proteins: a novel class of plant proteins with antifungal properties. Plant Journal 25(6) : 651-661.

Watkins, W.M. and Morgan, W.T.J. 1952. Neutralization of the anti-H agglutinin in eel serum by simple sugars. Nature 169 : 825-826.

Wilson, B., Abraham, G., Mamju, V.S., Mathew, M., Vimala, B., Sundaresan, S. and Nambisan, B. 2005. Antimicrobial activity of *Curcuma zedoaria* and *Curcuma malabarica* tubers. Journal of Ethnopharmacology 99(1) : 147-151.

Winter, H.C., Montafapour, K. and Goldstein, I.J. 2002. The Mushroom *Marasmius oreades* lectin is a blood group type B agglutinin that recognizes the galalpha 1,3Gal and gal α 1,3Galβ 1,4GlcNAc porcine xenotransplantation epitopes with high affinity. Journal of Biological Chemistry 277(17) : 14996-15001.

Wong, J.H., Chan, H.Y.E. and Ng, T.B. 2008. A mannose/glucose-specific lectin from Chinese evergreen chinkapin (*Castanopsis chinensis*). Biochimica et Biophysica Acta (BBA) - General Subjects 1780(9) : 1017-1022.

Xu, H.W., Dai, G.F., Liu, G.Z., Wang, J.F. and Liu, H.M. 2007. Synthesis of andrographolide derivatives: A new family of [alpha]-glucosidase inhibitors. Bioorganic and Medicinal Chemistry 15(12) : 4247-4255.

Yan, Q., Jiang, Z., Yang, S., Deng, W. and Han, L. 2005. A novel homodimeric lectin from Astragalus mongholicus with antifungal activity. Archives of Biochemistry and Biophysics 442(1) : 72-81.

Yenjai, C., Prasanphen, K., Daodee, S., Wongpanich, V. and Kittakoop, P. 2004. Bioactive flavonoids from *Kaempferia parviflora*. Fitoterapia 75(1) : 89-92.

Yenjai, C., Sutthanut, K., Srichairatanakool, S., Mungkun, N., Wongma, C., Mukdasai, S. and Kongkeaw, T. 2007. Further studies of bioactive flavonoid from *Kaempferia paviflora*. <u>KKU Science journal</u> 35(1) : 37-41.

Yogisha, S.H., and Raveecha, K.A. 2009. Alpha Glucosidase inhibitory activity of *Morus Alba*. <u>Pharmacologyonline</u> 1 : 404-409.

Yoshida, K., Hishida, A., Iida, O., Hosokawa, K. and Kawabata, J. 2008. Flavonol caffeoylglycosides as alpha-glucosidase inhibitors from *Spiraea cantoniensis* flower. Journal of Agricultural and Food Chemistry 56(12) : 4367-4371.

Young, N.M. and Oomen, R.P. 1992. Analysis of sequence variation among legume lectins. A ring of hypervariable residues forms the perimeter of the carbohydrate binding site. <u>Journal of Molecular Biology</u> 228 : 924-934.

APPENDICES

Appendix A

Solutions in Bradford Assay

A. Bradford stock solution

	Final concentration	Amount
Ethanol	95% (v/v)	100 ml
Phosphoric acid	88% (v/v)	200 ml
Serva Blue G		350 mg

B. Bradford working buffer

	Final concentration	Amount
Ethanol	95% (v/v)	15 ml
Phosphoric acid	88% (v/v)	30 ml
Bradford stock solution		30 ml
Distilled water		to 500 ml

Filter through Whatman No.1 paper, store at room temperature in brown glass bottle.
Useble for several week, but may need to be refilled.

ศูนย์วิทยทรัพยากร
จุฬาลงกรณ์มหาวิทยาลัย

Appendix B

Solutions in SDS-PAGE

The acrylamide, *N-N'*-methylenenbisacrylamide, TEMED, ammoniumpersulfate and SDS in this experiment are extremely hazardous. Hazardous materials should be weighted in a fume hood while wearing a disposables dust mask and using double latex gloves for all protocols.

I. Stock solutions

A. 2M Tris- HCl (pH 8.8), 100 ml

	Final concentration	Amount
Tris-base	2 M	24.2 g
Distilled water		50 ml
HCl		Adjust to pH 8.8
Distilled water		To 100 ml

Allow solution to cool to room temperature, pH will incrase.

B. 1M Tris-HCl (pH 6.8), 100 ml

	Final concentration	Amount
Tris-base	1 M	12.1 g
Distilled water		50 ml
HCl		Adjust to pH 6.8
Distilled water		To 100 ml

Allow solution to cool to room temperature, pH will increase.

C. 10% SDS (w/v), 100 ml

	Final concentration	Amount
SDS	10% (w/v)	10 g
Distilled water		To 100 ml

Store at room temperature

D. 50% glycerol (v/v), 100 ml

	Final concentration	Amount
100% glycerol	50% (w/v)	50 ml
Distilled water		To 50 ml

E. 1% bromophenol blue (w/v), 10 ml

	Final concentration	Amount
bromophenol blue	1% (w/v)	100 mg
Distilled water		To 10 ml

Filtration will remove aggregated dye.

II. Working Solutions

A. Solution A (acrylamide stock solution)

(30% acrylamide, 0.8% N,N' – methylenebisacrylamide)

	Final concentration	Amount
Acrylamide	30% (w/v)	29.2 g
N,N' – methylenebisacrylamide	0.8% (w/v)	0.8 g
Distilled water		To 100 ml

Solution can be stored for month in the refrigerator.

B. Solution B (4a separating gel buffer)

(2 M Tris-HCl, pH 8.8, 10% SDS)

	Final concentration	Amount
2 M Tris-HCl (see stock solution A)	1.5 M	75 ml
10% SDS (see stock solution C)	0.4%	4 ml
Distilled water		to 100 ml

Solution can be stored for month in the refrigerator.

C. Solution C (4x stacking gel buffer)

(1 M Tris-HCl, pH 6.8, 10% SDS)

	Final concentration	Amount
1 M Tris-HCl (see stock solution B)	0.5 M	50 ml
10% SDS (see stock solution C)	0.4%	4 ml
Distilled water		to 100 ml

Solution can be stored for month in the refrigerator.

D. 10% Ammonium persulfate

	Final concentration	Amount
Ammonium persulfate	10%	0.5 g
Distilled water		To 5 ml

Stable for month in a capped tube in the refrigerator.

E. Electrophoresis buffer

	Final concentration	Amount
Tris-base	25 mM	3 g
Glycine	192 mM	14.4 g
SDS	0.1% (w/v)	1 g
Distilled water		to 1000 ml

pH should be approximately 8.3

F. 5x Sample buffer

(1 M Tris-HCl, pH 6.8, 50% glycerol, 10% SDS, 2- mercaptoethanol)

	Final concentration	Amount
1 M Tris-HCl (see stock solution B)	60 mM	0.6 ml
50% glycerol (see stock solution D)	25% (v/v)	5 ml
10% SDS (see stock solution C)	2% (v/v)	2 ml
2-mercaptoethanol	14.4 mM	0.5 ml
Distilled water		to 1000 ml

Stable for week in the refrigerator or for months at -20°C.

III. Coomassie gel stain

	Amount
Coomassie blue R-250	1.0 g
Methanol	450 ml
Glacial acetic acid	100 ml
Distilled water	450 ml

IV. Coomassie gel destain

	Amount
Methanol	100 ml
Glacial acetic acid	100 ml
Distilled water	800 ml

V. Gel preparation

	Separating gel (10ml)	Stacking gel (4ml)
Solution A	5.0 ml	0.67 ml
Solution B	2.5 ml	-
Solution C	-	1.0 ml
10% ammonium persulfate	50 µl	30 ml
TEMED	5.0 µl	5.0 µl
Distilled water	2.5 µl	2.3 ml

10% Ammonium persulfate and TEMED are added in the last step because polymerization will be under way.

Appendix C

Calibration curve for protein determination by Bradford method

Y = 0.0155X
$R^2 = 0.9881$

A_{595} (nm)

BSA (μg)
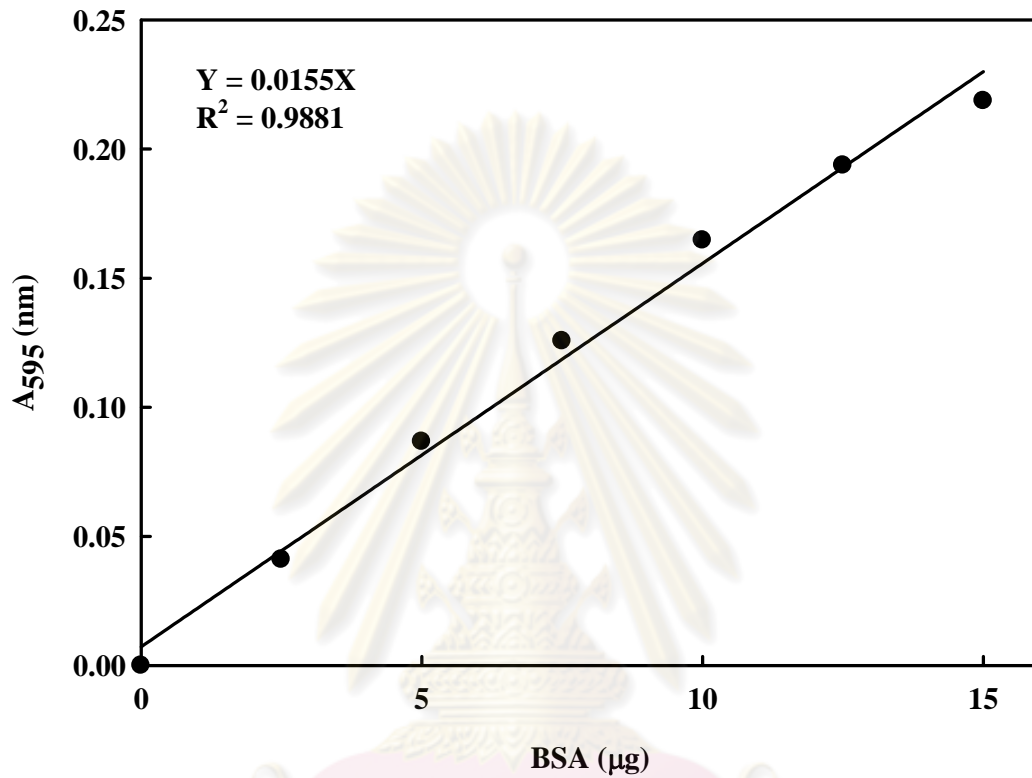
Appendix D

Calibration curve for carbohydrate content by Dubois method

$Y = 0.003X$

$R^2 = 0.9916$
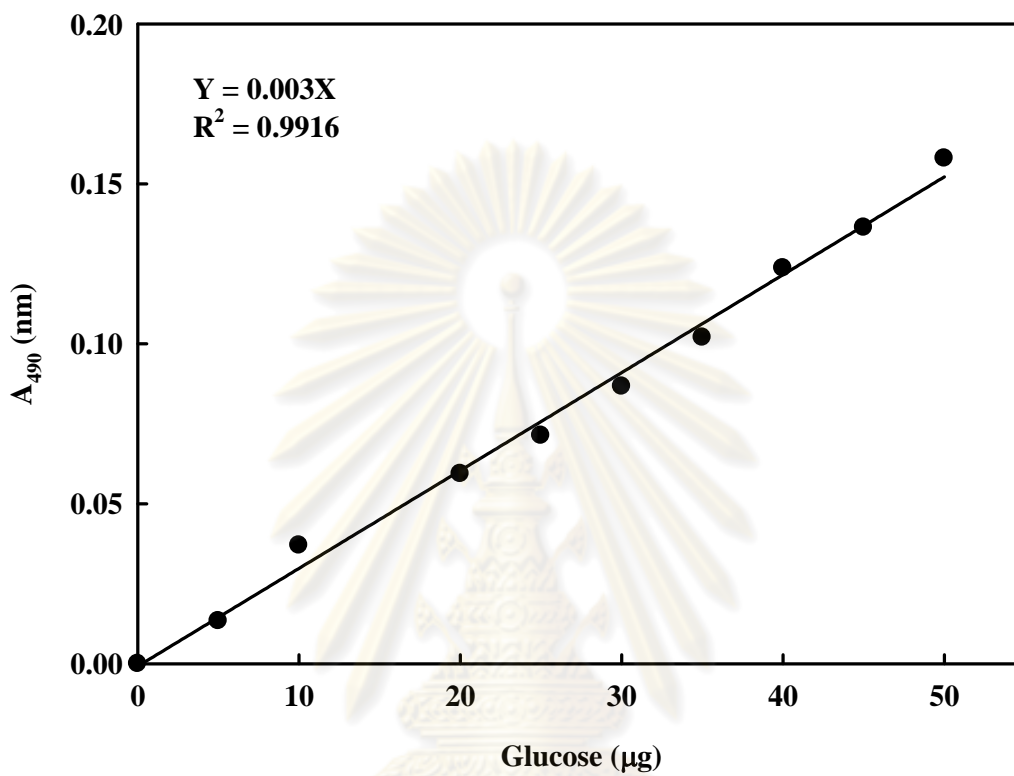
BIOGRAPHY

Miss Wichchulada Konkumnerd was born on June 27, 1983 in Samut Songkhram, Thailand. She graduated with a Bachelor Degree of Science from the Faculty of Engineering and Industrial Technology, Silpakorn University in 2005. She had been studies for a Master Degree of Science in Biotechnology, the Faculty of Science, Chulalongkorn University since 2007.

Academic presentation;

1). Konkumnerd, W., Karnchanatat, A. and Sangvanich, P. A Newly Thermostable Lectin from *Kaempferia parviflora* Wall. Ex Baker with Antifungal Activity. The 2nd BMB Conference: Biochemistry and Molecular Biology for Regional Sustainable Development. 7-8 May 2009. Faculty of Science, Khon Kaen Universtiy, Khon Kaen, Thailand.

2). Konkumnerd, W., Karnchanatat, A. and Sangvanich, P. A Newly Thermostable Lectin from *Kaempferia parviflora* Wall. Ex Baker with Antifungal Activity. The 4th Annual Symposium of the Protein Society of Thailand (PST). 26-28 August 2009. Chulabhorn Research Institute Conference Center. Bangkok, Thailand.