การทำให้บริสุทธิ์และลักษณะสมบัติของเลคตินจากเหง้าของขมิ้นชัน *Curcuma longa* Linn.

นางสาวปริยพร เพ็ชรนวล

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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PURIFICATION AND CHARACTERIZATION OF LECTIN FROM RIZOMES OF *Curcuma longa* Linn.

Miss Pariyaphon Petnual

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By	Miss Pariyaphon Petnual
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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

S. Hannongbua ..... Dean of the Faculty of Science

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

THESIS COMMITTEE

..... Chairman

(Associate Professor Amorn Petsom, Ph.D.)

Polkit Sangvanich ..... Thesis Advisor

(Associate Professor Polkit Sangvanich, Ph.D.)

Aphichart Karnchanatat ..... Thesis Co-Advisor

(Aphichart Karnchanatat, Ph.D.)

N. Ngamrojanavanich ..... Examiner

(Associate Professor Nattaya Ngamrojanavanich, Ph.D.)

Chantragan Phiphobmongkol ..... External Examiner

(Chantragan Phiphobmongkol, Ph.D.)

ปริยพร เพ็ชรนวล : การทำให้บริสุทธิ์และลักษณะสมบัติของเลคตินจากเหง้าของขมิ้นชัน *Curcuma longa* Linn. (PURIFICATION AND CHARACTERIZATION OF LECTIN FROM RHIZOMES OF *Curcuma longa* Linn.) อ. ที่ปรึกษาวิทยานิพนธ์หลัก : รศ.ดร.พลกฤษณ์ แสงวณิช, อ. ที่ปรึกษาวิทยานิพนธ์ร่วม: ดร.อภิชาติ กาญจนทัต, 81 หน้า.

ในงานวิจัยนี้ได้ทำการศึกษาลักษณะสมบัติของเลคตินจากเหง้าของขมิ้นชัน *Curcuma longa* Linn. โดยการนำเหง้าของขมิ้นชันมาสกัดด้วยสารละลายทริสบัฟเฟอร์ที่ค่าความเป็นกรดด่าง 7.2 จากนั้นนำโปรตีนมาทำให้บริสุทธิ์โดยการตกตะกอนด้วยเกลือแอมโมเนียมซัลเฟตอิ่มตัวที่ 80 เปอร์เซนต์ ทำเลคตินให้บริสุทธ์โดยเทคนิคโครมาโทกราฟีแบบสัมพรรคภาพด้วยคอลัมน์ ConA Sepharose เลคตินนี้ เมื่อใช้เทคนิคพอลิอะคริลาไมด์เจลอิเล็กโตรฟอเรซิสแบบเสียสภาพ ซึ่งเลคตินบริสุทธิ์ที่ได้มีน้ำหนักโมเลกุลประมาณ 17.3 กิโลดาลตัน และมีกิจกรรมการเกาะกลุ่มของเซลล์เม็ดเลือดแดงกับหมู่เลือดบีของคน กระต่าย หนูแรท หนูเมาส์ หนูตะเภา ห่าน และแกะ ตามลำดับ เลคตินเสถียรที่อุณหภูมิต่ำกว่า 50 องศาเซลเซียส เป็นเวลา 60 นาที และสูญเสียกิจกรรมอย่างสมบูรณ์ที่อุณหภูมิ 70 องศาเซลเซียสขึ้นไป ขณะที่เลคตินสามารถทนค่าความเป็นกรด-ด่างที่ 2 จนถึง 5 และเสถียรที่ ค่าความเป็นกรด-ด่างที่ 6 จนถึง 7 เลคตินมีความต้องการไอออนของแคลเซียม และแมงกานิสไอออนอย่างน้อย 50 มิลลิโมลาร์ ซึ่งจำเป็นสำหรับเสถียรภาพของโครงสร้าง และกิจกรรมของเลคติน การวิเคราะห์ลำดับกรดอะมิโนภายในโมเลกุลของเลคติน ด้วยการย่อยเลคตินด้วยทริปซิน แล้ววิเคราะห์ด้วยเครื่องแมสเปกโตรเมตรี ลำดับกรดอะมิโนที่พบ มีความคล้ายคลึงกับเลคตินในพืชตระกูล Fabaceae นอกจากนี้ เลคตินสามารถยับยั้งการเจริญเติบโตของราโรคพืช ได้แก่ *Exserohilum turcicum, Fusarium oxysporum* และ *Colectrotrichum cassicola* ที่ความเข้มข้น 47 จนถึง 94 ไมโครกรัม สามารถยังยั้งการเจริญเติบโตของจุลินทรีย์ด้วยค่าความเข้มข้นน้อยสุดที่สามารถยับยั้งของเชื้อ *P. aeruginosa, S. aureus, B. subtilis, C. albican* และ *E. coli* เรียงลำดับจากน้อยไปมาก ดังนี้ คือ 0.002, 0.005, 0.011, 0.046 และ 0.09 มิลลิกรัมต่อมิลลิลิตร และค่าความเข้มข้นการยับยั้งการทำงานของแอลฟา- กลูโคซิเดสที่ 50 เปอร์เซนต์ เท่ากับ 0.0166 มิลลิกรัมต่อมิลลิตร

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PARIYAPHON PETNUAL: PURIFICATION AND CHARACTERIZATION OF LECTIN FROM RIZOMES OF *Curcuma longa* Linn. THESIS ADVISOR: ASSOC. PROF. POLKIT SANGVANICH, Ph.D., THESIS CO-ADVISOR: APHICHART KARNCHANATAT, Ph.D, 81 pp.

A lectin was purified from the rhizomes of turmeric (*Curcuma longa* L.) by aqueous extraction, precipitation with 80% saturation ammonium sulfate, and affinity chromatography on ConA Sepharose with Methyl-α-D-glucopyranoside mediated elution. The enriched preparation had a specific activity of 64,566 HU/mg protein for a yield of 41.2% total protein. The molecular weight of this lectin was estimated by SDS-15% (w/v) PAGE to be 17.3 kDa. It has hemagglutinating activity against human blood group B, as well as rabbit, mouse, rat, guinea pig, geese and sheep erythrocytes, but no such activity was detected against human blood groups A, AB and O. The pH optimum of this lectin's hemagglutination activity is between pH 6 - 7, and it is stable up to 40 °C but is totally inactivated after exposure to 70 °C for 30 min. The hemagglutination activity was stimulated by Ca^{2+} and Mn^{2+}at less than 50 mM, but not by Mg^{2+}, Fe^{3+}, Hg^{2+}, Co^{2+} and EDTA. The amino acid sequence of an internal fragment of this purified *C. longa* rhizomal lectin had a similarity to the sequence of the phytohemagglutinin precursor from the plant legume lectin family. The purified *C. longa* lectin, at a concentration of 47 and 94 μg/0.3 cm^2 disc showed antifungal activity against *Exserohilum turicicum*, *Fusarium oxysporum* and *Colectrotrichum cassiicola*. The lowest to highest minimal inhibitory concentration (MIC) values of 0.002, 0.005, 0.011, 0.09 and 0.046 mg/ml were obtained against the four bacterial species, *P. aeruginosa*, *S. aureus*, *B. subtilis*, and *E. coli*, plus the yeast *C. albicans*, respectively, for the purified lectin preparation, along with a high α-glucosidase inhibitory activity with an IC_{50} of 8 μg/ml.

Field of study:.....Biotechnology.....Student's signature.....Pariyaphon Petnual.....

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Co-advisor's signature.....Aphichart Karnchanatat.....

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LIST OF ABBREVIATIONS

%	percentage
^{O}C	degree celsion
µg	microgram
µl	microliter
A	Absorbance
BSA	Bovine serum albumin
cm	centimeter
Da	Dalton
EDTA	Ethyllenediamine tetraacetic acid
g	gram
hr	hour
kDa	kilodalton
l	liter
LC-MS-MS	Liquid Chromatography-Mass Spectrometry-Mass Spectrometry
M	Molar
mA	Miliampere
mg	Miligram
min	minute
ml	milliliter
mM	milimolar
MW	Molecular weight
NaCl	Sodium Chloride
PAGE	Polyacrylamind gel electrophoresis
rpm	revolution per minute

RT	Room temperture
SDS	Sodium dodecyl sulfate
TEMED	N,N,N’,N’-tetramethyl ethylenediamine
Tris	Tris(hydroxymethyl)amiomethane
U	Unit activity
V	Volt
V/V	Volumn by volumn
W/V	Weight/volumn

CHAPTER I

INTRODUCTION

Abundantly plants contain carbohydrate-binding proteins that are called lectins, agglutinins or phytohemagglutinins as enzymes bind substrates and inhibitors, antibodies bind antigens. Lectins are protein or glycoprotein specific with least two sugar-binding sites per unit. These molecules are nonimmune origin and bind reversibly with specific sugar lectins were precipitate polysaccharide, glycoprotein and glycolipids (Goldstein *et al*., 1980) because lectin as polyvalent or univalent carbohydrate-binding oligomeric proteins. The interaction of lectins at all surface sugar and sugar binding site on lectin molecules they are usually noncovalant, related hydrogen bonding, hydrophobic interactions and van der Waal's forces. In plant lectins were found widely distributed in the Leguminoseae such as kidney beans (Shi *et al*., 2007), *Parkia Ja Vanica* beans (Utarabhand and Akkayanont 1995), *Dolichos lablab* (Lavanya *et al*., 2006). The biological activities of lectins had displayed including anti-tumor (Pusztai 1998), immunomodulatory (Abdullaev and Mejia 1997), anti-fungal (Rubinstein *et al*., 2004), anti-human immundoficiency virus (HIV) (Herre *et al*., 2004), and anti-insect activities (Barrientos and Gronenborn 2005).

Turmeric (*Curcuma longa* L.) belongs to the Zingiberaceae family which noteworthy members like ginger, cardamom and galangal. The genus *Curcuma* consists of hundreds of species of plants that cover rhizomes and underground root like stems. Turmeric is specially importance to humans that discovery its rhizome powder, when added to various food preparations, preserves their freshness and sense of a characteristic flavors. Turmeric belongs to a group of aromatic spices, had been originally used as a food additive in curries to improve the storage condition palatability and preservation of food. Turmeric are grown in warm, rainy regions of the world such as China, India, Indonesia, Jamaica and Peru (Govindarajan 1980) including southern and southeastern tropical Asia.

In medically, turmeric has been used internally as a stomachic, tonic and blood purified and externally in the prevention and treatment of skin diseases (The Wealth of India, 2001). Traditional Indian medicine claims the use of its powder against biliary disorders, anorexia, coryza, cough, diabetic wounds, hepatic disorder,

rheumatism and sinusitis (Ammon *et al*., 1992). In the part turmeric has been used in clinical testing but in present its has proved the bactericidal properties of turmeric and now its use is more than being merely cosmetic (Khanna 1999). In this study has objective of this work is to undertake the purification and characterization of lectin from rhizome of *C. longa* and study on its biological activity. It is noted the activity of lectin from *C. longa* has not yet been reported.

CHAPTER II

REVIEW OF THE LITERATURES

2.1 The discovery of lectins

The lectin had been found by Stillmark in 1888 (Stillmark 1988) that the toxicity of castor bean extracts it presence of a proteinaceous hemagglutinating factor called ‘‘ricin’’. In 1898 Elfstrand introduced the term ‘‘hemagglutinin’’ as a common name for all plant proteins that clump blood cells (Elfstrand 1898). In the following decade, lectins had been reported for the first time the presence of nontoxic lectins in the legumes *Phaseolus vulgaris*, *Pisum sativum*, *Lens culinaris*, and *Vicia sativa* (Landsteiner and Raubitschek 1907). In the subsequent years, many nontoxic lectins were discovered and it became clear that plant lectins are widespread in the plant kingdom. 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. Only in 1952 lectins were shown that the agglutination properties of lectins are based on a specific sugar binding activity (Watkins and Morgan 1952).

Goldstein showed definition of lectins are carbohydrate binding proteins of nonimmune origin which agglutinate cells and/or precipitate glycoconjugates (Goldstein *et al.*, 1980). The nomenclature of plant lectins is very diverse. Since the early discovery plant lectins were usually designated by a trivial name, often a composite of an abbreviation of the (scientific) name of the source plant followed by the suffix -in (e.g., ricin from *Ricinus communis*). Other plant lectins were designated by three-letter abbreviations derived from the plant or plant material in which the protein was found (e.g. WGA, wheat germ agglutinin; PNA, peanut agglutinin).

2.2 Distribution of lectins

2.2.1 Bacterial lectins

Among the bacteria found to react with lectins are a variety of Gram-positive and Gram-negative organisms as well as the specialized forms, mycobacteria and mycoplasma. Hamada et al. reported that 13 of 15 strains of *Streptococcus mutans* tested were agglutinated by Con A (Hamada *et al*., 1977). The study surface features on membranes used by Con A in many organism such as Con A agglutinates a variety of Gram-negative bacteria, especially *Salmonella* spp. Certain R mutants of *Escherichia coli* and *Salmonella* spp. are also reactive with this lectin (Kato *et al*., 1979). The site of lectin binding is exposed sugars on the lipopolysaccharide (LPS) molecule. Another Gram-positive of *Staphylococcus aureus* is agglutinated by a lectin from the horseshoe crab distinct from limulin (Gilbride and Pistole 1979). Furthermore inhibition studies using D-Glcose and D-galactose (Percin *et al*., 2009), and precipitin reactions with polysaccharides derived from these organisms, indicated that different ligands on streptococcal cells from different serogroups were responsible for the interactions with Con A (Kashket and Guilmette 1975)

2.2.2 Fungal lectins

Over 40 years ago Sumner & Howell reported that cells of *Saccharomyces cerevisiae* (baker's yeast) were markedly agglutinated by lectin (James and Stacey 1936). Since then, numerous reports on the interaction of Con A with yeast and fungi have appeared. The major binding site for Con A in yeast or fungi is mannan in particular the α-linked D-Man residues. Although most studies on lectin *Saccharomyces* interactions have used Con A, at least one report indicates that WGA also binds to these yeast cells, presumably to the disaccharide di-*N*-acetylchitose found in the cell wall (Horisberger and Rosset 1976). Con A binds to *Candida albicans* (Barkai-Golan and Sharon 1978; Cassone *et al*., 1978; Jansons and Paktor 1977), *Candida utilis* (Barkai-Golan and Sharon 1978; Horisberger and Rosset 1977; Horisberger *et al*., 1976; Horisberger and Rosset 1977), and other *Candida* spp. (Barkai-Golan and Sharon 1978; Cassone, *et al*., 1978; Guillot *et al*., 1974; Jansons and Paktor 1977) via the mannan located in the cell walls of these microorganisms.

Many of the reports noted above involving *Saccharomyces* spp. have been similarly carried out with *Candida* spp.; the results are comparable for both yeast forms. Con A reacts with blastospores of *C. albicans*, again via mannan receptors. Cells from which mannan was extracted reacted poorly if at all with this lectin (Cassone *et al*., 1978). In *Fusarium* spp. binding of RCA is seen only after trypsinization. KOH treatment also exposes receptors for this lectin on *F. roseum*, but not on *F. solani*, adding support to the idea that these related forms differ in their cell wall organization

2.2.3 Algal lectins

Although molecules with agglutinating activity have been found in a large number of marine algae, only a few have been studied in detail. A few species that have been investigated are *Pilota plumose, Agardhelia tenera, Cystoclonium purpuream, Gracilaria verrucosa, Palmaria palmate, Fucus vesciculosum, Codium fragile* (Fabregus and Carracedo 1989).

2.2.4 Viral lectins

The viral lectins have been discussed in detail by Sharon and Lis Influenza virus possesses a glycoprotein molecule in the membrane that mediates binding (Sharon and Lis1989). The carbohydrate binding site from a pocket located in a domain of the lectin protruding from the membrane and is composed of the amino acids that are largely conserved in many strains of viruses. Vlasak reported that the differences in the specificity of different strains of influenza viruses to bind to enzymatically modified erythrocytes carrying terminal *N*-acetyl α-neuraminic acid attached to D-galactose either by (2→3) or (2→6) linkages could be correlated with the species origin of the virus (Vlasak 1988). Thus, human isolates preferentially agglutinated resialylated erythrocytes containing an α-Neu Ac-(2→6)-D-Gal sequence, whereas the equine and avian isolates associate with α-Neuu Ac-(2→3)-D-Gal.

2.2.5 Animal lectins

The occurrence of hemagglutinins in animals was noted quite early, almost all in invertebrates or lower vertebrates, but till the middle of the 1970s, only the three of these mentioned above (of eel, snail and horseshoe crab) were isolated and characterized. The first of the animal lectins shown to be specific for a sugar (L-fucose) was from the eel (Watkins and Morgan 1952). The isolation in 1974 of the first mammalian lectin, the galactose-specific hepatic asialoglycoprotein receptor, was an outcome of the investigation by Gilbert Ashwell at the N.I.H. together with Anatol G. Morell at the Albert Einstein Medical School, New York, of the mechanisms that control the lifetime of glycoproteins in blood circulation (Hudgin *et al*. 1974). At the same time Vivian Teichberg from our department reported (Teichberg *et al*., 1975) the isolation from the eel of the first member of the family of the β-galactose-specific lectins, indicate as galectins (Barondes *et al*., 1994). Since the beginning of the 1980s the number of purified animal lectins also started to grow fast, largely to the advent of recombinant techniques.

2.2.6 Plant lectin

Lectins have been found in a wide variety of species almost every major taxonomical classification of flowering plants (Allen and Brilliantine1969; Mialonier *et al*., 1973; Toms and Western 1971). Many plants and their individual tissues have been routinely screened for lectins by measuring the ability of their extracts to agglutinate erythrocytes. Although this hemagglutination assay has been of great value in detecting lectins, it is at best semiquantitative; it will not detect inactive or monovalent lectin, nor will it provide accurate estimates of lectin if an endogenous receptor for that lectin is present in the extract. The assay can at times yield false positive results because of nonspecific hemagglutination caused by lipids (Tsivion and Sharon 1981) or by polyphenols such as tannins (Kriipe 1956 and Makela 1957) that are often abundant in plant tissues. It is therefore advisable to verify positive hemagglutination data by inhibiting the activity with specific sugars or by isolating the lectin.

The carbohydrate specificities and structures of lectins from a large variety of plants have been studied in considerable detail (Goldstein and Hayes 1978; Goldstein

and Poretz 1985; Lis and Sharon 1981). In general, lectins from plants within particular taxonomical groups have distinctive properties that distinguish them from lectins of less closely related plants. It is important to note that the lectins used in these comparisons represent the most abundant and therefore most intensively studied lectins in the plants of these families. These lectins are not all derived from homologous tissues. These differences in origin must be remembered in interpreting these comparisons since, as is discussed below, it is possible that different tissues within the same plant may contain different lectins. This reservation does not apply to comparisons of lectins obtained from homologous tissues of plants within the same family. Homologies within two of these families, the Graminaceae and Leguminoseae, are discussed in further detail below.

Graminaceae: The lectin from monocotyledonous plants is the wheat germ agglutinin, which is a 36,000 molecular weight dimer of identical protein subunits linked by interchain disulfide bonds (Nagata and Burger 1974; Rice and Etzler 1974). The complete amino acid sequence of this lectin has recently been determined (Wright *et al*., 1984). This lectin has a specificity for oligomers of β (1→4)-*N*-acetyl-oglucosamine (Allen *et al*., 1973). Lectins with similar specificities and molecular properties have been isolated from rye (Peumans *et al*., 1982b) and barley embryos (Mishkind *et al*.,1983; Peumans *et al*., 1982b). Indeed, these lectins are so similar that they can undergo subunit exchange to form heterodimers (Peumans *et al*., 1982a).

Leguminoseae: The seeds of legumes are particularly rich in lectins, and many of these lectins have been characterized extensively (Goldstein and Hayes 1978; Goldstein and Poretz 1985; Lis and Sharon 1981). As this review was prepared, the complete amino acid sequences of concanavalin A (Edelman *et al*. 1972), favin (Cunningham *et al*., 1979), and lectins from lentil (Foriers *et al*., 1981), sainfoin (Kouchalakos *et al*., 1984), *Phaseolus vulgaris* (Hoffman *et al*., 1982), soybean (Hemperly *et al*., 1982; Vodkin 1983), and pea (Higgins *et al*., 1983) have been determined. In addition, the NH_2 terminal amino acid sequences of at least 15 other legume lectins are available (Strosberg *et al*., 1983). Comparisons of these sequences have shown extensive homologies, particularly among those lectins from plants within the same tribes. It is clear that these lectins have been conserved during evolution of the legumes and that the homologies in their NH_2 terminal amino acid

sequences reflect the taxonomical relationships of the plants in this family (Foriers *et al*., 1977; Foriers *et al*., 1979; Strosberg *et al*., 1983).

2.3 Isolate and purification of lectin

Purified lectins are essential for establish their molecular properties and are highly desirable for their many applications. In the past, lectins have been obtained solely from native sources, but they can now be produced also by recombinant techniques

2.3.1 From natural sources

Isolation of a lectin begins commonly with extraction of the tissue or organ in which it is present. This is simple in the case of plants, especially their seeds (Figure. 2.1) (Goldstein and Poretz 1986; Rudiger 1993). The seeds are ground and the meal obtained is extracted with a neutral buffer. Often it is advisable to pre-extract the dry meal with an organic solvent, such as petroleum ether, to remove colored materials derived from the seed coat and lipids that may be present in large amounts. Animal tissues are either homogenized directly in the extraction buffer or the tissue is extracted first with acetone to remove water and lipids. The extraction buffer should preferably contain protease inhibitors to prevent degradation of the lectin during purification, and, in the case of membrane bound lectins, a detergent as well.

Preliminary fractionation of the crude extract (e.g., by ammonium sulfate precipitation) is often done to obtain a protein fraction devoid of other constituents (e.g., polysaccharides in the case of plants). Final purification is achieved by affinity chromatography on a suitable adsorbent. A wide variety of affinity adsorbents, to suit any taste or purse, have been described in the literature and many of them can be purchased ready-made These include polysaccharides such as Sephadex, a polymer of glucose employed for the purification of concanavalin A and pea lectin agarose (or Sepharose), a polymer of galactose, for the purification of the lectins from castor bean; acid-treated Sepharose for the purification of SBA; and chitin, a polymer of *N*-acetylglucosamine, for the purification of WGA. In the absence of readily available polysaccharides, use can be made of adsorbents consisting of carbohydrates or glycoproteins as such, or in the form of a synthetic derivative, that are covalently

attached to an insoluble carrier. For instance, lactose coupled to Sepharose is the reagent of choice the purification of the lectins from peanut, eel electric organ or calf heartmuscle. *N*-Acetylglucosamine bound to the same support serves for the purification of potato lectin and WGA, whereas immobilized porcine AH blood type substance is employed for the purification of the blood type A specific DBL and HPA. When working with lectins of an uncommon specificity, adsorbents have to be tailor made, as for example Sepharosebound asialoglycophorin for the purification of the blood type *N*-specific for lectin from *Vicia graminea*.

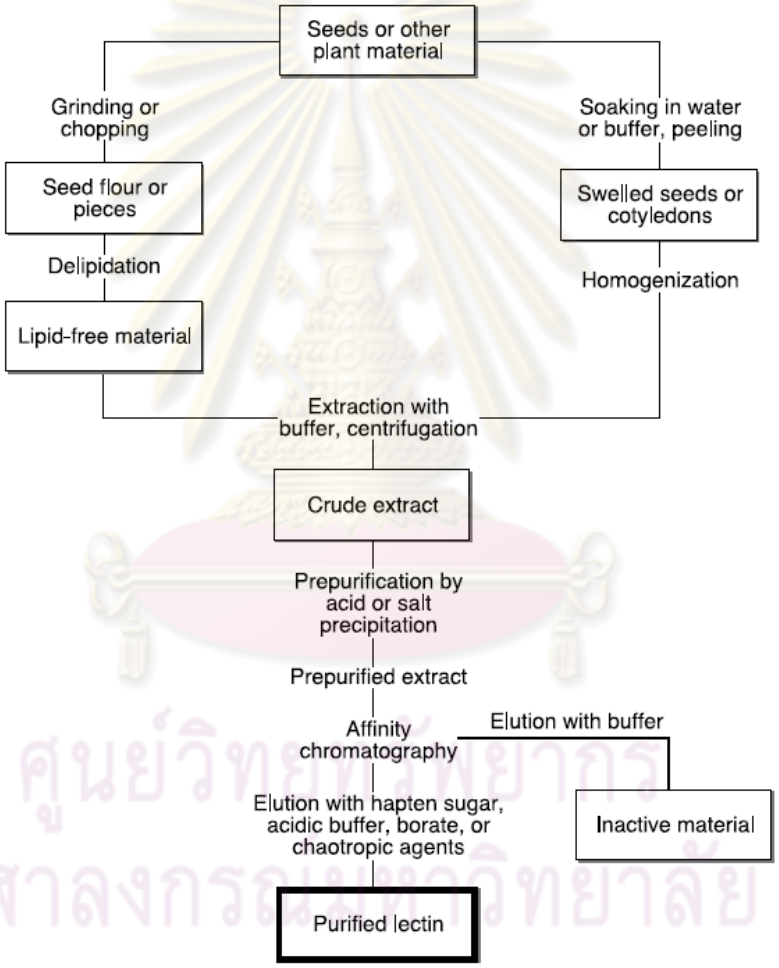

Figure 2.1 Scheme for lectin purification. reproduced with permission from Rudiger (Rudiger 1993)

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2.3.2 By recombinant techniques

An alternative approach for the preparation of lectins has been made possible by the advent of recombinant DNA technology. It is based on the isolation of the cDNA or genomic DNA of the lectin, its insertion into a suitable vector and expression in an appropriate host cell. Isolation of the cDNA requires knowledge of at least part of the primary sequence of the lectin itself or of a structurally similar one. By this technique, several plant lectins, among them of pea (Stubbs *et. al*., 1986; Van Eijsden *et al*., 1992), *Erythrina corallodendron* (Arango *et al*., 1993), peanut (Sharma and Surolia 1994) and *Griffonia simplicifolia* (Zhu *et al*., 1996) have been expressed in *E. coli*. Expression of plant lectins was also achieved in other systems, e.g. WGA in *Saccharomyces cerevisiae* (Nagahora *et al*., 1992)**,** PHA and GNA in *Pichia pastoris* (Raemaekers *et al*., 1999), PNA in insect cells (Kumar *et al*., 1999) and SBA in monkey cells (Adar *et al*. 1997); (for a more complete listing of recombinant plant lectins) (Streicher and Sharon 2003).

2.4 Biological activities of lectins

2.4.1 Assays for hemagglutinating

The agglutinating activity of lectins is usually measured with erythrocytes, using the serial 2-fold dilution technique, the endpoint is determined either visually or spectrophotometrically. Since the visual determination is a subjective operation, significant variations in the results may occur even in the same laboratory. The speetrophotometrie method is more accurate, but also more laborious, requires larger amounts of material, and is used only in a limited number of laboratories. An additional difficulty in comparing hemaggiutinating activities of lectins stems from the fact that different types of erythrocytes are used in different studies, e.g. human A, B, or O type, or rabbit, either untreated or after treatment with trypsin (the latter treatment increases markedly the sensitivity of the cells to agglutination). However highly purified lectins will, under suitable conditions, exhibit hemagglutinating activity at concentrations as low as 0.1μg/ml (Irvin 1976).

2.4.2 Lectins with anti-viral activity

Diverse plant lectins exhibit a markedly inhibitory effect on animal and human viruses in vitro. For example, the chitin-binding lectin from stinging nettle (*Urtica dioica*) and the mannose-specific agglutinins from Amaryllidaceae and Orchidaceae species are potent inhibitors of the infection of target cells with retroviruses such as the human immunodeficiency virus and cytomegalovirus (Balzarini *et al*., 1991; Balzarini *et al*., 1992).

2.4.3 Lectins with anti-bacterial activity

Recent observations with regard to the binding of plant lectins to components of the bacterial cell wall peptidoglycans (such as muramic acid, *N*-acetylmuramic acid, *N*-acetylglucosamine and muramyl dipeptides) revealed that seed lectins from several legume species strongly interact with these bacterial surface carbohydrates (Ajouba *et al*., 1994). Evidently, the observation that legume seed lectins can recognize and bind to the bacterial cell wall does not imply that such an interaction occurs *in vivo* and certainly does not prove that these lectins are involved in the protection of the seedlings against bacteria.

2.4.4 Lectins with anti-fungal activity

In 1975 Mirelman et al. was found wheat germ agglutinin (WGA) can be inhibits spore germination and hyphal growth of *Trichoderma viride* and interferes with the synthesis of chitin (Mirelman *et al*., 1975). A novel mannose-binding lectin was purified from rhizomes of *Ophiopogon japonicus* was showed antifugal activity in three phytopathogenic fungi namely *Gibberella saubinetii* and *Rhizoctonia solani* (Tian *et al*., 2008)

2.4.5 Lectins with anti-insect properties

In 1976 Janzen and his collaborators observed the phytohemagglutinin (PHA) from black beans *(Phaseolus vulgaris)* exhibited an insecticidal action on the bruchid beetle *Callosobruchus maculatus* (cowpea weevil) (Janzen *et al*., 1976). Interestingly,

the R-amylase inhibitor which is a potent inhibitor of insect R-amylases it is structurally related to PHA, although it has no carbohydrate-binding properties (Moreno *et al*., 1990; Moreno and Chrispeels 1989). Moreover, besides the R-amylase inhibitor, some wild accessions of beans contain arcelin, another PHA-like protein, which was shown to be toxic for the bean weevil *(Zabrotes subfasciatus)* (Osborn *et al*., 1988). It appears, therefore, that beans do not rely on lectins for their defence against insects but rather on lectin related proteins.

2.4.6 Plant lectins with toxicity for higher animals

Many plant lectins are toxic to animal cells but not all. Lectins consumed in the diet may be innocuous; if it had been denatured by cooking and proteolytically digested upon consumption. However raw lectins can have deleterious effects, since uncooked lectins are extremely stable to proteases. For example consumption of raw beans not alters the intestinal microflora and results in gastrointestinal dysfunction. Raw soybean lectin and wheat-germ agglutinin induce release of cholecystokinin, suggesting that these types of lectins may have direct effects on gastrointestinal function and growth. While the experiment of Pereira et al. was extraction and purification of cassava leaf agglutinin and injected intraperitoneal via in mice. The results showed no deaths or any adverse effects were observed after 120 h (Pereira *et al*., 2008).

2.5 Sugar binding activity and specificity of plant lectins

Broadly reveal, lectins can be divided into those that bind monosaccharides as well as oligosaccharides, and those that recognize oligosaccharides only (Wu *et al*., 2001). It is noteworthy that almost all saccharides recognized by lectins are typical constituents of animal cell surfaces. This is perhaps a reflection of the method commonly used for lectin detection (Tsivion and Sharon 1981), as a result of which lectins recognizing sugars not present on erythrocytes might have been overlooked.

2.5.1 Mannose/glucose

A lectin with specificity for mannose and glucose residues has been isolated in crystalline form the fava bean (*Vicia laba*) by a procedure which included absorption to Sephadex. It has a molecular weight of 50,000 Da and appears to be a tetramer made of two subunits of 18,000 Da and two subunits of 9,000 Da. These studies determine amino acid sequence and three-dimensional structure of lectin were similar with structural features of Concanavalin A (Irvin 1976)

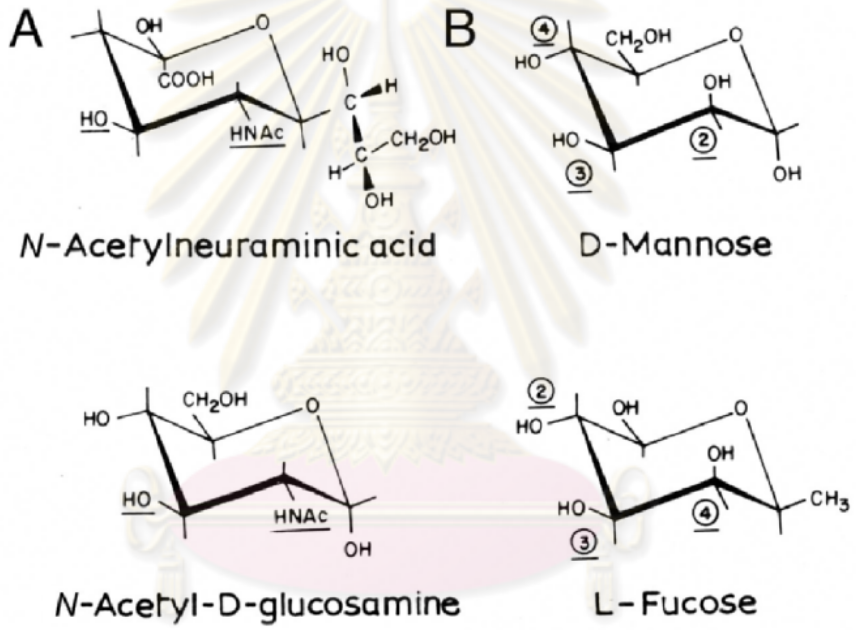

Figure 2.2 Common structural features of *N*-acetylneuraminic acid and *N*-acetylglucosamine (A) and of mannose and fucose (B). Similarity of *N*-acetylglucosamine and *N*-acetylneuraminic acid at positions C-2 (acetamido) and C-3 (hydroxyl) of the pyranose ring is observed when the sialic acid molecule is suitably rotated. Rotation of the fucose molecule by 180o allows superimposition of its ring oxygen, 4-OH, 3-OH and 2-OH with the ring oxygen, 2-OH, 3-OH and 4-OH of mannose, respectively. Groups that thus occupy the same positions in space are underlined. (Sharon and Lis 1993)

2.5.2 Galactose/*N*-acetylgalactosamine

As mentioned, lectins interacts with galactose or *N*-acetylgalactosamine such as the lectin from the corn coleoptyle. It is a glycoprotein had molecular mass under non-denaturing conditions was 88.7 kDa And had carbohydrates that constituted 12% of the total weight comprised galactose, mannose, and *N*-acetyl-D-glucosamine (Martinez-Cruz *et al*., 2001). In 2003 Konoay *et al*. were found *Erythrina speciosa* seeds can be specific with D-galactose and had two identical subunits of molecular mass was 27.6 kDa include the lectin was a neutral carbohydrate content of 5.5% (Konozy *et al*., 2003). *N*-acetyl-D-galactosamine-specific lectin isolation from *Glycine max* L. Merrill SA88 them were found the soybean lectin consists of four subunits it had molecular weight of each 30,000 Da in one-step purification with high purity and high yield (about 90% recovery from the crude extract) by use Poly (hydroxypropyl methacrylate-glycidyl methacrylate) beads were as an affinity matrix and *N*-acetyl-D-galactosamine (GalNAc) was as an affinity ligand (Percin *et al*., 2009).

2.5.3 Fucose

Aleuria aurantia lectin (AAL) is a commercially available lectin that is known for its high affinity for α-1,6-fucosylated oligosaccharides and it is widely used to estimate the extent of α-1,6-fucosylation on glycoproteins and to fractionate glycoproteins. For research a novel probe for core fucose from *Aspergillus oryzae* L-fucose-specific lectin (AOL) has strongest preference for the alpha 1,6-fucosylated chain among α-1,2-, α-1,3-, α-1,4-, and α-1,6-fucosylated pyridylaminated (PA)-sugar chains. These results suggest that AOL is a novel probe for detecting core fucose in glycoproteins on the surface of animal cells (Matsumura *et al*., 2007). Furthermore, *Lotus tetragonolobus* lectin is a fucose-specific legume lectin. It is a homotetramer composed of four legume lectin domains was 27,800 Da (Moreno *et al*. 2008).

2.5.4 Sialic acids

Most of Sialic acid-specific lectins was found in invertebrates such as those from the Indian horseshoe crab (Mohan *et al*., 1982), marine crab *Scylla serrata* (Mercy and Ravindranath 1992), lobster, tunicalase, fungus *Hericium arinaceum* (Kawagishi *et al*., 1994) and leaves of mulberry (Ratanapo *et al*., 1998). A lectin from the white shrimp *Litopenaeus setiferus* (LsL) hemolymph is a heterotetramer of two 80 kDa and two 52 kDa subunits, *N*-acetylated sugars, such as GlcNAc, GaINAc, and NeuAc, were the most effective inhibitors of the LsL hemagglutinating activity. Desialylation of erythrocytes or inhibitory glycoproteins abolished their capacity to bind LsL, confirming the relevance of sialic acid in LsL-ligand interactions (Alpuche *et al*., 2005). In 2009 the *Phaseolus coccineus* lectin (PCL) specificity towards sialic acid showed the molecular mass of 30 kDa consisting of homodimer subunits. Moreover the purified PCL was devoid of antifungal activity against *C. albicans* and *P. italicum*, but markedly inhibited the growth of *H. maydis*, *Rhizoctonia solani*, *G. sanbinetti*, *S. sclerotiorum* while the same concentration of PCL decrease the 50% hemagglutinating activity was inhibited by sialic acid it suggesting a significant correlation between sialic acid-specific site and its bi-functional bioactivities (Chen *et al*., 2009).

2.6 Lectins in edible plants

Many lectin-containing plants are common constituents of the diet of humans and farm animals. Since lectins are known to act on cells in a variety of ways, such as agglutination, mitogenic stimulation and killing, and they are often resistant to heat and proteolytic enzymes, including those of intestinal bacteria, the effects of consumption of these proteins deserve special consideration.(Liener 1986) For many years it has been known that they occur in legumes such as soybeans, kidney beans, lima beans, mung beans, lentils, garden peas and peanuts that are a major food source for humans and animals in one part of the world.

Although lectin containing foods are frequently consumed in cooked or otherwise processed form, such treatments may not always be adequate to completely inactivate the lectins present. Thus, lectins have been detected in roasted peanuts

(Wang *et al*., 1999). Slow cooking of beans, without boiling, does not always eliminate lectin activity as observed with kidney beans cooked for 11 hr at 82 ^{o}C or for 5 hr at 91 ^{o}C. The stability of plant lectins in the stomach is evidenced, for example, by the finding that when concanavalin A, PHA or WGA were intragastrically administered into rats between 50 and 90% of the lectin was recovered after 1 hr from the stomach by homogenizing the tissue in phosphate-buffered saline containing the appropriate specific sugar (Pusztai and Bardocz 1995). Moreover, in the few experiments with humans that ate lectin-containing foods, namely tomatoes (Kilpatrick *et al*., 1985), red kidney beans (Pusztai *et al*., 1989) or peanuts, either raw or roasted (Wang *et al*., 1999) the lectins have not only withstood the acidity and the proteolytic enzymes of the intestinal tract, but a significant proportion of the amount ingested has reached the circulatory system with unimpaired hemagglutinating and immunological activities. In rodents, a diet containing lectins provoked intestinal and systemic immune responses to these proteins (Gomez *et al*., 1995). Furthermore, human serum was found to contain antibodies to the lectins of peanut, soybean and wheat germ (Tchernychev and Wilchek 1996)

2.7 Family of Zingiberaceae

The plants members of Zingiberaceae family are notice characterized by their tuberous rhizomes its have strong aromatic and medicinal properties. It is usually found as ginger, and exists in about 50 genera and 1,300 species worldwide, distributed mainly in South and Southeast Asia (Wu and Larsen 2000). The medicinal functions for treatment of diseases such as diarrhea, coryza, dermatosis disorders and rheumatism are also widely mentioned in traditional remedies (Ammon *et al*., 1992; Charles and Charles 1992; Miquel *et al*., 2009; Skrzypezac-Jankun *et al*., 2000). Many essential oils had accessories of Zingiberaceae plants including terpenes, alcohols, ketones, flavonoids, carotenoids and phytoestrogens (Habsah *et al*., 2000; Mau *et al*., 2003; Suhai 2006). For example, the water extract of *Zingiber officinale* showed 6-Gingerol, and was mostly found in the rhizome in concentrations of 130-7,138 ppm. The major compound in Curcuma is curcumin, which has concentrations as high as 38,000 ppm in species (Suhai 2006). Besides Curcuminoids, gingerols and kava pyrones isolated from Zingiberaceous plants have been reported for their

biological activities in antifungal, antioxidant, insecticidal, and anti-inflammatory activities (Sirat 1994; Sirat *et al*., 1996; Sirat and Liamen 1995).

2.8 Species of *Curcuma longa* Linn

Curcuma longa L. rhizomes or tumeric or chemical extract called curcumin. They are perennial herb belonging to the ginger family. Turmeric measures up to 1 m high with a short stem and tufted leaves. The distribution of tumeric was a tropical plant native to southern and southeastern tropical Asia especially South Asia mainly India such as Andhra Pradesh and Tamil Nadu. India produces 600,000 tons of turmeric annually which is 75% of world production of 800,000 tons. Turmeric extract is a yellow-orange powder that is insoluble in water and ether but soluble in ethanol, dimethylsulfoxide, and acetone. Three Chemical constituents of *C. longa* are 77%curcuminI, 17% curcumin II (Demethoxycurcumin) and 3% curcumin III (Bis-Demethoxycurcumin: less active). The structure of curcumin $C_{21}H_{20}O_6$ it has a melting point of 183 ^{o}C and a molecular weight of 368.37 g/mol. Curcumin is stable at acidic pH but unstable at neutral and basic pH, under which conditions it is degraded to ferulic acid and feruloylmethane.

Figure 2.3 Rhizome of *Curcuma longa* Linn.

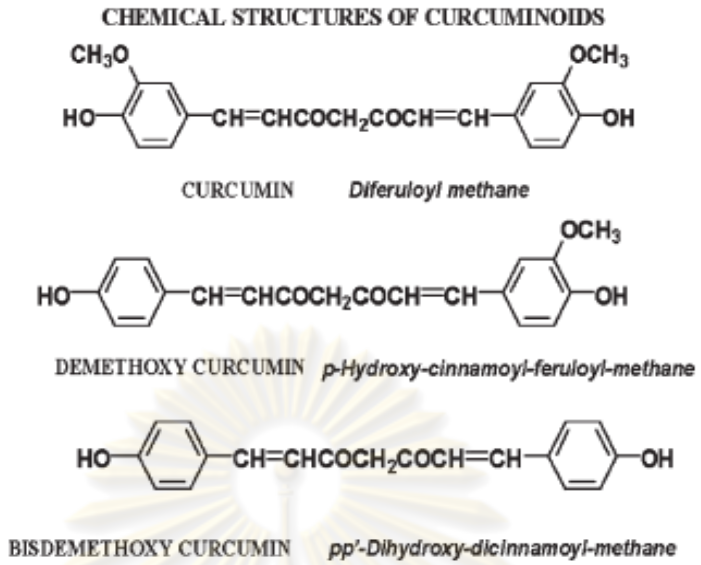

Figure 2.4 Chemical structures of curcumin, demethoxy curcumin and bisdemetohxy curcumin (Maheshwari *et al*., 2005)

Tumeric has been widely used for consume in varies centuries such as added to various food preparations, preserves their freshness and imparts a characteristic flavour. Specific nutritional facts of tumeric in 100 g of edible turmeric contain 65 Kcal of total energy, 1.2 g of protein, 1.4 g of lipid, 11.4 g of carbohydrate, 9 g of calcium, 41 g of phosphorous, 2.3 g of iron, 0.02 g of vitamin B1, 0.03 g of vitamin B2, 1.3 g of niacin, and 12 g of vitamin C. (Oonmettaaree 2005). Report of the pharmacological activities of curcumin including anti-inflammatory (Satoskar *et al*., 1986; Srimal and Dhawan 1973), anti-cancer (Kuttan *et al*., 1985), anti-oxidant (Sharma 1976; Toda *et al*., 1985), wound healing (Sidhu *et al*., 1998) and anti-microbial effects (Negi *et al*., 1999).

Some research has demonstrated curcumin's ability to inhibit carcinogenesis at three stages: tumor promotion, angiogenesis and tumor growth. Curcumin suppresses mitogen-induced proliferation of blood mononuclear cells, inhibits neutrophil activation and mixed lymphocyte reaction and also inhibits both serum-induced and platelet derived growth factor (PDGF)-dependent mitogenesis of smooth muscle cells (Huang *et al*., 1992). It has also been reported to be a partial inhibitor of protein kinase (Aggarwal *et al*., 2003; Liu *et al*., 1993). The other salient feature of

turmeric/curcumin is that despite being consumed daily for centuries in Asian countries, it has not been shown to cause any toxicity (Ammon and Wahl 1991).

CHAPTER III

EXPERIMENTAL

3.1 Chemicals

Acarbose (Glucobay-Bayer, Germany)
Acetic acid (Merck Ag Darmstadt, Germany)
Acrylamind (Plusone Pharmacia Biotech, Sweden)
Agar (Difco, USA)
Alpha Glucosidase (Sigma, USA)
Ammonium persulfate (Plusone Pharmacia Biotech,Sweden)
Ammonium sulfate (Merck Ag Darmstadt, Germany)
Barium chloride (Merck Ag Darmstadt, Germany)
Beef extract (Difco, USA)
Bis-acrylamide (Promega, USA)
Bovine serum albumin (Sigma, USA)
Bromophenol Blue (USB, USA)
Calcium Chloride (Merck Ag Darmstadt, Germany)
Cobalt chloride (Fluka, Switzerland)
Con A sepharose 4B (Sigma, USA)
Copper sulfate (Fluka, Switzerland)
Coomassie Brilliant Blue G-250 (USB, USA)
Copper sulfate (Fluka, Switzerland)
D-Glucose (Fluka, Switzerland)
Ethylenediaminetetraacetic acid, EDTA (Sigma, USA)
Ethanol (Merck Ag Darmstadt, Germany)
Ferric chloride (Merck Ag Darmstadt, Germany)
Glycerol (Fluka, Germany)
Glycine (Sigma, USA)
Hydrochloric acid (J.T. Baker, USA)
Manganese Chloride (Sigma, USA)
Magnesium sulfate (Fluka, Switzerland)

Malt extracts (Difco, USA)

Mercury chloride (Merck Ag Darmstadt, Germany)

Methanol (Merck Ag Darmstadt, Germany)

Methy-α-D-glucopyranoside (Sigma, USA)

2-mercaptoethanol (Merck Ag Darmstadt, Germany)

N, N'-methylene-bis-acrylaminde (Plusone Pharmacia Biotech, Sweden)

p-nitrophenol (sigma,U.S.A)

p-nitrophenol-α-D-glucopyranoside, PNPG (Sigma, USA)

Peptone (Pronadisa-Conda, Spain)

Phenol (Merck Ag Darmstadt, Germany)

Phosphoric acid (Mallinckrodt Chemicals, USA)

Sodium acetate (Merck Ag Darmstadt, Germany)

Sodium carbonate (Merck Ag Darmstadt, Germany)

Sodium chloride (Merck Ag Darmstadt, Germany)

Sodium Dodecyl sulfate (Plusone Pharmacia Biotech, Sweden)

Sodium hydroxide (Merck Ag Darmstadt, Germany)

Standard Molecular Weight Marker (Sigma, U.S.A)

Sulfuric acid (J.T. Baker, USA)

Tetramethylethylenediamine, TEMED (Plusone Pharmacia Biotech, Sweden)

Tris (USB, U.S.A)

Yeast extract (Bio Basic, Canada)

Zine Sulfate (Merck Ag Darmstadt, Germany)

3.2 Equipment

Affinity Column Chromatography (AKTA prime, Amersham Pharmacia Biotech, Sweden)
Autoclave (Isuzu, Seisakushu Co., Ltd., Japan)
Autopipette (Pipetman, Gilson, France)
Dialysis bag (Snake Skin Dialysis Tubing, Pierce, U.S.A)
Electrophoresis unit (Hoefer mini VE, Amersham Pharmacia Biotech, Sweden)
Freeze dryer (Labconco, U.S.A)
High Speed Refrigerated Centrifuge (Kubota 6500, Japan)
Hot air oven (Memmert, Germany)
Hot plate stirrer (HL instrument, Thailand)
Laminar Flow (Safety Lab, Asian Chemical and Engineering Co., Ltd., Thailand)
Microcentrifuge (Biofuge pico Heraeus, Kendro, Germany)
Orbital Shaker (OS-10 Biosan, Latvia)
pH meter (Mettler Toledo, U.S.A)
Pipette tips (Bioline, U.S.A)
Power Supply (EPS 301 Amersham Phamacia Biotech, USA)
Refrigarated incubator shaker (Innova 4330, USA)
Sonicate (DHA-1000, Branson, U.S.A)
Spectrophotometer (Synergy HT Biotek, USA)
Speed vacuum centrifuge (Heto-Holten, Denmark)
96-well microtter plate (Costar, USA)
Vortex mixer (Vortex-Genie2, Scientific Industries, U.S.A)
Water Bath (NTT-1200 Tokyo kikakikai, Japan)

3.3 Plant material

Rhizomes of *C. longa* purchased from the local market (Bangkok,Thailand). A voucher specimen (BK60689) is deposited at the Bangkok Herbarium (BK) of The Plant Variety Protection Division, Department of Agriculture, Thailand.

3.4 Blood Sample

Human blood cells (A, AB, B and O) of healthy donors obtained were kindly offered by the National Blood Center, Thai Red Cross Society. Rabbit, mouse, rat, guinea pig, goose and sheep blood cells were purchased from the National Laboratory Animal Center, Mahidol University. The blood sample were centrifuged at 5000 rpm for 5 min at 4^{o}C then supernatant discard and washed in Tris-buffered saline (TBS) replete in 3 time and either used immediately or stored for up to 4 weeks at 4^{o}C

3.5 Microorganisms

- Tested microorganisms

Bacillus subtilis ATCC6633 (Gram positive bacteria)

Staphylococcus aureus ATCC 25923 (Gram positive bacteria)

Escherichia coli ATCC 25922 (Gram negative bateria)

Pseudomonas aeruginosa ATCC 27853 (Gram negative bateria)

Candida albican ATCC 10231 (fungi)

Exserohilum turicicum (fungi)

Fusarium oxysporum (fungi)

Colectrotrichum cassiicola (fungi)

- Positive Control

Penicilin 1mg/ml

Amplicilin 1mg/ml

Ketoconazole 200 mg/ml

- Growth of microorganisms

B.subtilis, *S.aureus*, *E.Coli* and *P. aeroginosa* were growth on nutrient broth (Alpuche, J., et al., 2005) and nutrient agar (NA) at 37^{o}C and were maintained in NA at 4^{o}C. Their stocks were kept in NB with 10% sterile glycerol at -70^{o}C.

C.albican was grown on yeast-malt medium (YM) and yeast-malt broth (YMB) at 30^{o}C. It was maintained and kept as above with YM and YMB with 10% sterile glycerol.

3.6 Lectin Purification

All of the procedures were performed at 4^{o}C, unless otherwise stated.

(i) Protein extraction and $(NH_4)_2SO_4$ precipitation. Rhizomes of Curcuma longa (400g) were peel off and cut. After that it was homogenized in 20 mM Tris-HCl buffer, pH 7.2, containing 0.15 M NaCl using a blender. The homogenate was extracted with isolation buffer (1L) by stirred overnight then filtered through cheesecloth and centrifuged at 8,000 rpm for 30 min. The supernatant were added with ammonium sulfate to give 80% saturation, and then stirred overnight. The suspension was collected by centrifugation at 8,000 rpm for 30 min and then supernatant was discarded. The precipitate was dissolved in 20 mM Tri-HCl buffer, pH 7.2 and then desalted by dialysis with the same buffer over 24 hour.

(ii) Affinity chromatography. The crude solution was applied to a Con A sepharose (1.6 x 20 cm) installed in an AKTA prime instrument equilibrated with buffer A (20 mM Tris-HCl buffer, pH 7.2, containing 0.15 M NaCl). The column was washed with 100 ml of buffer A (2.0 M Methyl α-D-glucopyranoside) to remove unbound proteins. And then bound proteins were eluted with Buffer B at a flow rate of 1.5 ml/min. The bound protein was dialyzed against distilled water for 24 h at 4 ^{o}C and freezes dry.

3.6.1 Determination of protein concentration

The protein which was obtained during purification process was determined the concentration using Bradford assay (Bradford 1976). Bovine serum albumin (BSA) was used as the standard. For the calibration curve, the solution of 1 mg/ml of BSA were pipetted triplicate volumes at 2.5, 5, 7.5, 10, 12.5, 15, 17.5, 20 µL into microcentrifuge tubes, and then was adjusted volume to 100 µL with 20 mM Tri-HCl buffer, pH 7.2. Pipette the 100 µl of same buffer was used as blank. Then 10 µl standard solution and protein sample was added to 96 well-microtiter plate and then 100 µl of Bradford reagent was added to all well. Finally, the samples and standards solution was measured the absorbance at 595 nm. The calibration curve was prepared and the concentration of protein was calculated.

3.7.4 Carbohydrate content

The phenol sulfuric acid method was originally described as a nonspecific quantitative test for carbohydrate which that solution gives a brown color (Dubois *et al*., 1956). This method can be used for the quantitative colorimetric micro determination of monosaccharide and their polymerization product, such as oligosaccharides and polysaccharide. Glucose was used as the standard. For the calibration curve, pipette triplicate volumes of 5, 10, 15, 20, 25, 30, 35, 40, 45 and 50μL of 1 mg/ml glucose standard solution into test tubes, and adjust the volume to 1 ml with sterile water while sample was add 200 μl in test tube. To the each test tube were added consecutively 1 ml of 5% phenol solution and 10 ml of concentrated H_2SO_4 rapidly. All additions to the test tubes were made with mixing well. Hold on the solution in room temperature about 10 minutes and bring to ice box about 5 minutes. Then add 200 μl of standard solution and sample solution to 96 well-microtiter plate. Finally Measure the absorbance at 490 nm of the samples and standards solution compare with 200 μl of sterile water into a plate for the reagent blank, plot calibration curve x-axis is glucose concentration and y-axis is absorbance.

3.7.5 Determination of protein pattern on native-PAGE

Proteins from each step of purification were subjected for analysis of native protein pattern according to the method of Bollag (Bollag *et al*., 1996).

Non-denaturating gel electrophoresis. The gel was carried out with 7.5% separating gel, and 5.0% stacking gel. Tris-glycine buffer pH 8.3 was used as the electrode buffer. The electrophoresis was run from cathode toward anode at a constant current of 20 mA per slab at room temperature in a Mini-Gel Electrophoresis unit. After electrophoresis, protein in the gel were visualized by coomassie Blue R-250 staining and destaining.

Coomassie blue staining and destaining. The gel was staining solution consist of 0.1% (w/v) coomassie Blue R-250 in 10% (v/v) acetic acid and 45% (v/v) methanol. Destaining was performed by immersing the gel in de-staining solution consist of 10% (v/v) acetic acid and 45% (v/v) methanol) followed by several changes of destaining solution unit the background was clear.

3.7.6 Hemagglutination assay

Fresh rabbit erythrocyte had been washed and make to 3% rabbit erythrocyte by 20 mM Tris-HCl buffer, pH 7.2. Hemagglutination assay was carried out in a microtiter plate following a two-fold serial dilution method. The lectin solution (50 µl) was place in the first well and serially dilute into the successive well with the same buffer. Then, 50 µl of 3% rabbit erythrocyte suspension was added to the entire well. Hemagglutination was visualized in the plate after 1 h of incubate at 37^{o}C (Wong *et al*., 2006). Titer define as the reciprocal of the highest dilution exhibiting hemagglutination, was considered equal to one hemagglutination unit. Specific activity was started as the number of unit per mg of lectin (Tipthara *et al*., 2007).

3.8 Biochemical characterization lectin from *C. longa*

3.8.1 Molecular weight determination

The purified lectin was subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) for molecular mass determination. The gel was prepared with 0.4% in 15% separating gels (pH 8.8) and 5% stacking gel (pH 6.8). Tris-glycine buffer pH 8.3 containing 0.1% SDS was used as the electrode buffer. The SigmaMarker^{TM} wide molecular weight range used as the protein marker. Discontinuous SDS-PAGE in reducing condition (in the present of ß-mercaptoethanol) was performed according to the procedure of Laemmli (Laemmli, U.K., 1970). Sample to be analyzed were treated with sample buffer and boiled for 5 min before application to the gel. Electrophoresis was run from the cathode to anode at constant current of 20 mA per slab at room temperature in a Mini-gel Electrophoresis unit. High and low molecular weight standards were used to determine the subunit molecular weight standards were used to determine the subunit molecular weight of the enzyme. After electrophoresis, protein in the gel were visualized by staining with Coomassie Blue R-250 and de-stain in solution of 10%methnol 10% acetic acid in water until band appeared

3.8.2 Blood group specificity

Human blood of groups A, B, AB and O collected from four donors and animal blood (rabbit, mouse, rat, guinea pig, goose and sheep) were used for checking the blood group specificity of the lectin. A serial twofold dilution of the lectin solution (50 µl) in microtiter U-plates was mixed with 50 µl of Tris-HCl buffer (pH 7.2) and add 3% suspension of human and animal blood cells in each well. The results were read after about 1 h when the blank had fully sediment. The hemagglutination titer, defined as the reciprocal of the highest dilution exhibiting hemagglutination, was reckoned as one hemagglutination unit or one titer. Specific activity is the number of hemagglutination units per mg protein.

3.8.3 Effect of temperature on lectin activity

The lectin solution was heated in a multiblock heater from 25 ^{o}C (RT) to 100 ^{o}C, at intervals of 10 ^{o}C, for a period of 30 min. The residual hemagglutinating activity was assayed after adjusting the temperature to 4^{o}C compare with control, which was the sample solution incubate at room temperature was considered to be 100% activity. At least three replicates were done for each test to confirm the results.The inactivation kinetics of the protein was performed using lectin solution heating from 40 to 70 ^{o}C at intervals of 10 ^{o}C in a multiblock heater. Agglutination activity was checked every 10 min using a 50µl aliquot over a period of nearly 2 hour after adjusting the temperature to 4^{o}C. An aliquot taken before heating served to estimate as control, which was considered to be 100% activity.

3.8.4 Effect of pH on lectin activity

Incubating the protein with buffer from pH 2-14 assessed the pH stability and pH optimal of the lectin. The buffer used were 20mM glycine-HCl (pH 2-4), 20mM Sodium acetate (pH 4-6), 20mM Potassium phosphate (pH 6-8), 20mM Tris-HCl (pH 8-10) and 20mM glycine-NaOH (pH 10-12). The purified lectin was mixed to difference buffer (lectin: buffer = 1:1 v/v). After being left for 1 hour at room temperature, the samples were adjusted back to pH 7.2 with 20 mM Tris-HCl pH 7.2 (sample : Tris-HCl = 1:4) and assay for agglutinating activity compare with control,

which was the lectin solution mixed with Tris-HCl buffer pH 7.2 and left standing for 1 hour at room temperature was considered 100%.

3.8.5 Effect of metal ion

The effect of metal ion and chelating agent on hemagglutinating activity was investigated. The purified lectin (1 mg/ml) was incubated for 24 hour in a various concentrations (12.5, 25, 50, 100 mM) of metal solution; Hg^{2+}, Ca^{2+}, Co^{2+}, Mn^{2+}, Mg^{2+}, Fe^{2+} and EDTA with continuous shaking. Agglutination assay was performed as mentioned earlier.

3.8.6 Internal amino acid sequence of lectin by LC–MS/MS

The internal amino acid sequence of the purified lectin from *C. longa* was performed by in-gel digestion of the protein and sequencing of the different peptides by mass spectrometry. The ion spectra were analyzed and the sequence determined. The analysis was performed at the Genome institute, National Center for Genetic Engineering and Biotechnology, Pathumthani, Thailand. Sequence comparison of internal peptides of lectin with those of the lectin family was performed using individual peptides. Peptide matching from sample mass spectra was based on an accuracy of ±1 Da peptide. Amino acids were matched to the SWISSPROT data.

3.9 Other biological activity testing

3.9.1 Alpha-glucosidase inhibitory activity

α-glucosidase activity assay was developed according to Boonmee (Boonmee *et al*., 2007). α-glucosidase was assayed using 50mM Sodium acetate buffer at pH 5.5 and 1mM *p*-nitrophenyl-α-D-glucopyranoside (PNPG) 20 µl was used as substrate. The concentration of the enzyme was 1 Unit/ml in each incubate (20 µl) in 0.077 mg/ml of purified lectin in 20mM Tris-HCl buffer at pH 7.2 and incubate at 37^{o}C for 10 min. 20 µl of 1mM PNPG was then added to the mixture to initiate the enzyme reaction. The reaction was incubated at 37^{o}C for 30 min and stopped by adding 100 µl of 1M Na_2CO_3. α-Glucosidase activity was determined by measuring release of the yellow *p*-nitrophenol at 400 nm. One unit of α-glucosidase is defined as the amount enzyme liberating 1.0 µmole of *p*-nitrophenol (PNP) per minute under the conditions specified. 6.8 mg/ml acarbose was used as the positive control in this study.

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

Abs = Absorbance at 405 nm
Control = no sample in experiment
Blank = buffer

Half Inhibition Concentrations (IC_{50}) of the extracts were determined by constructing a dose-response curve and examined the concentrations of concentration that inhibited 50% of enzyme activity. IC_{50} is less corresponding to show high α-glucosidase inhibition. (Tunsaringkarn *et al*., 2008)

3.9.2 Assay of antifungal activity

The assay of *C.longa* lectin for antifungal activity toward *Exserohilum turicicum, Fusarium oxysporum,* and *Colectrotrichum cassiicola* was carried out in 90x15 mm petri plates containing 10 ml potato dextrose agar. After the mycelial colony had developed, sterile blank filter paper disks (0.625 cm in diameter) at a distance were placed 1 cm away from the rim of the mycelial colony. The samples

were dissolved in 20mM Tris–HCl buffer pH 7.2 containing 0.15 M NaCl. The plates were incubated at 27°C until mycelial growth had enveloped peripheral disks containing the control and had formed crescents of inhibition around the paper disks containing samples with purified lectin. Petri dish was incubated at 25 ^{o}C for 3 day-period, at the end of which 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.

3.9.3 Assay of antibacterial activity by Minimum inhibition concentration

3.9.3.1 Preparation of bacteria

B.subtilis, *S.aureus* and *E.Coli* from stock were streaked on NA and incubated at 37^{o}C for 24 hr. Then, their were picked up 5-6 fresh colonies, inoculated into 50 ml of fresh NB and shaking flask at 250 rpm, 37^{o}C for 6hr.

C.albican was grown as above on YM at 30^{o}C for 24 hours and in 50 ml YMB which shaking flash at 250 rpm, 30^{o}C for 6 hours.

3.9.3.2 Assay of antibacterial activities by MIC

The inoculated of bacteria were prepared from 6 hours broth cultures and suspensions were adjusted to McFarland standard turbidity number 0.5. The purified lectin (50 µl) was diluted in serial 2-fold dilutions in 96-well microtiter plate with 50 µl of broth. The initial concentration was the highest lectin in tested solution in first well. Then, add 50 µl of bacteria in each well. The final volume in each well was 150 µl. The broth media were used as negative controls. Amplicillin and Penicillin (1mg/ml) were used as positive controls to determine the sensitivity of tested solutions. The plate was covered with a sterile plate sealer and incubates for 24 hours at 37^{o}C and 30^{o}C for *C.albican*. Next, 600 nm absorbance were indicated growth of organisms.

CHAPTER IV

RESULTS AND DISCUSSION

4.1 Purification of lectin from *C. longa* rhizomes

The present study reports the purification and characterization of a lectin from the rhizomes of *C. longa*. Following homogenization and overnight extraction of *C. longa* rhizomes in TBS, and clarification by cheese cloth filtration and centrifugation to give a crude rhizome homogenate, 80% saturation ammonium sulphate fractionation reduced the total protein yield some 3.5-fold (28.6% yield) but slightly increased the total lectin activity (1.07-fold) resulting in a 3.7-fold increase in the total specific activity (Table 4.1). The paradoxical increase in total lectin activity (as hemagglutination activity) to above that of the starting level, may simply reflect removal of inhibitors or competing agents, in addition to simple experimental variations, but nevertheless was only a slight increment.

Further purification of the crude ammonium sulphate cut extract to enrich for lectins by ConA Sepharose column based affinity chromatography further reduced the total protein level some 63.8-fold, for a decrease in the total hemagglutination activity of only 2.6 fold, giving a 24.7-fold increase in the specific activity for this stage (Table 4.1). Since the unbound protein fractions in TBS were devoid of hemagglutination activity, likely indicating that the lectin was fully adsorbed to the matrix, this apparent 2.6-fold decrease in lectin hemagglutination activity may reflect the incomplete elution of lectin from the ConA Sepharose matrix in the column by the methyl α-D-glucopyranoside competitor, but the possibility of co-purification of methyl α-D-glucopyranoside with the lectin, removal of required metal cation cofactors or other denaturing actions cannot be excluded, since lectin levels were monitored by hemagglutination activity.

Regardless, the recovered ConA Sepharose bound lectin was eluted as a single peak after desorption with TBS supplemented with 0.5 M methyl α-D-glucopyranoside (Figure 4.1), and this was used in subsequent further studies as the enriched lectin preparation, and was shown to be purified *C. longa* lectin (see below). The protein from each step of purification was analyzed for purity and protein pattern

by native-PAGE (Figure 4.2), where after ConA Sepharose affinity chromatograpy a single protein band on the native-PAGE gel was observed, indicating that the purified lectin obtained from the ConA sepharose column should be a relatively pure lectin protein. The amount of *C. longa* lectin, the activity and specific activity were shown in Table 1. Therefore, assuming both purification to homogeneity and a constant relationship between hemagglutination activity and total lectin mass, the total yield of *C. longa* lectin was 0.12 mg/g (wet weight) rhizomes which accounted for about 0.45% (w/w) of the total protein in the crude rhizome extract. The purified lectin was found to contain 5.2% (w/w) carbohydrate content, which is about the same as the lectin from *Helianthus tuberosus* L. tubers, which were reported to have a 5.3% (w/w) equivalent of carbohydrate (Suseelan *et al*., 2002).

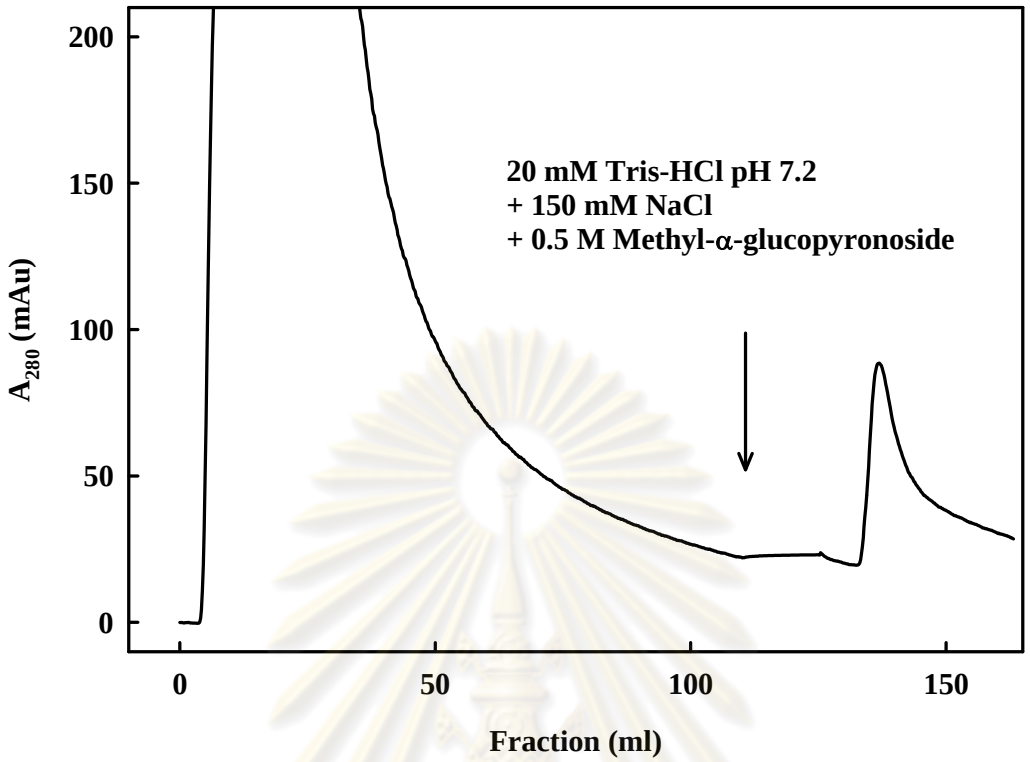

Figure 4.1 Affinity chromatogram of *C. longa* rhizome lectin on a ConA Sepharose column equilibrated and then washed with TBS. Lectin was then eluted with TBS containing 0.5 M Methyl α-D-glucopyranoside, as described in the methods section (start of elution indicated by the arrow).

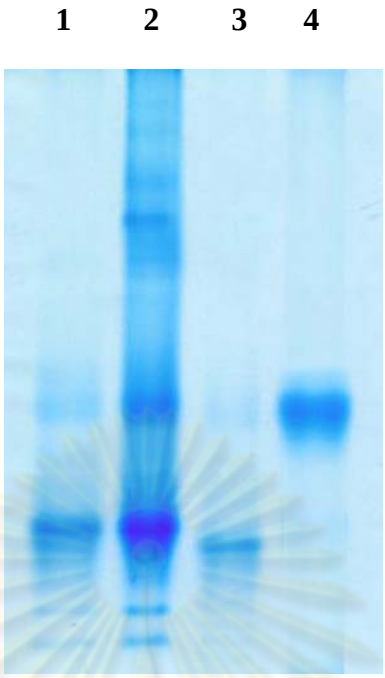

Figure 4.2 Coomassie blue stained non-denaturing PAGE of the *C. longa* rhizome lectin from each step of purification where lanes 1 - 4 show 20 μg of total protein from (1) the crude extract (homogenate), (2) the 80% ammonium sulphate precipitated and kept fraction (3) the non bound ConA sepahrose fraction discarded and (4) the bound fraction kept.

Modern isolation procedures generally employ affinity chromatography on an insoluble carbohydrate derivative (Lis and Sharon 1980; Goldstein and Hayes 1978) Such insoluble, naturally occurring, or chemically modified substances, such as insoluble hog gestic musin (Etzier and Kabat 1970), chitin (Shankar *et al*., 1976), arabinogalactan (Majumdar and Surolia 1978), sephadex (Wang et al., 1974) and agarose or sepharose, have also been employed as affinity matrics for purification of interesting lectins. For example, the mannose-glucose specific lectins from the seeds of the tepary bean (*Phaseolus acutifolius*) and mulberry, *Morus* sp. (Rosales: Moraceae), seeds were purified by a ConA Sepharose based affinity chromatography column (Richard *et al*., 1990; Absar *et al*., 2005). Another example is the lectin from ground elder (*Aegopodium podagraria*) rhizomes which could not be purified by Gal-NAG-Sepharose, but was purified using affinity chromatography of erythrocyte membrane proteins immobilized on cross-linked agarose (Peuman *et al*., 1985).

Table 4.1 Purification of the lectin from the rhizomes of *C. longa*.

Purification step	Total protein (mg)[a]	Total activity (HU)[b]	Specific activity (HU/mg)[c]	Yield (%)	Purification (fold)[d]
Crude extract	10,931	7.68×10^6	702.6	100	1
80% $(NH_4)_2SO_4$ precipitation	3132	8.19×10^6	2,616	106.7	3.7
Con A Sepharose (bound fraction)	49.1	3.17×10^6	64,566	41.2	91.9

[a] Crude protein extract from 400 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.

4.2 Molecular weight determination

The potential purity of the *C. longa* rhizomal lectin extract was evaluated at each step of the purification using SDS-PAGE under reducing conditions (Figure 4.3). The ammonium sulfate precipitated lectin showed many protein bands of a low molecular weight, with only a slight band at ~17 - 18 kDa. After the ConA-sepharose affinity purification step, a significant increase in the intensity of the 17.3 kDa band (estimated size) was seen, and only this band, suggesting a high degree of likely purity. The estimated mass (or subunit mass) for this lectin of ~17.3 kDa is in agreement with the previously published biochemical characteristics for other plant lectins, which reside within the 17 to 20 kDa range (Trifonova *et al*., 2004; Gaidamashvili *et al*., 2004)

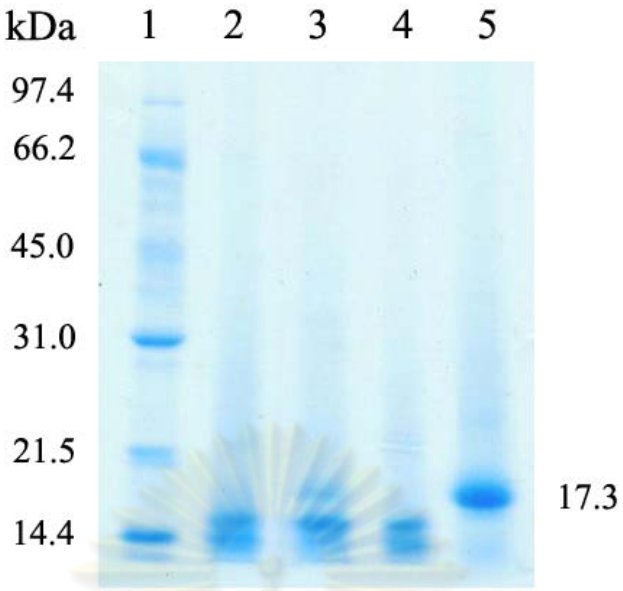

Figure 4.3 Reducing SDS-PAGE analysis of the *C. longa* rhizome lectin purification where lanes 2 - 5 show 20 μg of total protein. Lane 1, molecular weight standards; Lane 2, the crude extract (homogenate); Lane 3, 80% saturation ammonium sulphate precipitated (kept) fraction; Lane 4, the non-bound ConA-sepahrose fraction; Lane 5, the eluted ConA sepahrose-bound fraction (enriched lectin preparation).

4.3 Assay for hemagglutinating activity

Examination of the hemagglutination ability of the purified *C. longa* rhizomal lectin preparation with erythrocytes from different species (human, rabbit, mouse, rat, guinea pig, geese and sheep) revealed some interesting observations (Table 4.2). With respect to binding to human erythrocytes, the purified *C. longa* rhizomal lectin showed a moderately strong hemagglutination ability when tested against human blood group B, but, in contrast, no activity against the other three blood groups (A, AB and O), and thus shows a marked human blood group specificity. Moreover, the minimum concentration required to cause agglutination (MCA) of human blood group B cells (0.333 mg /ml) was much lower than that for the *Clitoria ternatea* lectin at 1.132 mg/ml (Naeem *et al*., 2007). The lectin from the rhizomes of *Urtica dioica* also shows no apparent human blood group-specific discrimination (Peumans *et al*., 1983), whilst that from *Galactia lindenii* seeds displayed a lower specific titer for human blood group A than that for groups B and O (Almanza *et al*., 2004). This difference in

the agglutination activity between groups may be due to the nature of the different glycoprotein(s) protruding on the erythrocyte cell surfaces, which are weakly or not totally recognized by the lectin (Oliveria *et al*., 2002).

However, outside of human erythrocytes, the purified *C. longa* rhizome lectin was a more potent agglutinin for, in order, native rat, rabbit, sheep and guinea pig erythrocytes (MCA = 0.01 - 0.08 mg/ml), with a weaker but significant avidity (hemagglutination activity) seen against geese and mouse erythrocytes (Table 4.2). These results, with the agglutination of native mouse, rat, guinea pig, and sheep RBCs, seem to be a novel character for the *C. longa* rhizome lectin as no other report concerning any lectin from other *Curcuma* species is currently available. In contrast, no agglutination was reported against mouse, cow, horse, dog and sheep erythrocytes for the lectin from *Galactia lindenii* seeds (Almanza *et al*., 2004), whilst variable hemagglutination, with a relatively higher potency against rabbit erythrocytes, was observed for that the lectin from *Pisum sativum* (Sitohy *et al*., 2007).

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

Blood type	MCA (mg/ml)
Human A	NA
Human B	0.333
Human AB	NA
Human O	NA
Rabbit	0.020
Mouse	0.665
Rat	0.010
Guinea pig	0.083
Geese	0.166
Sheep	0.042

The concentration of *C. amarissima* lectin used in this assays was 0.05 mg/ml and was serially 1:1 (v/v) diluted. Data shown are the mean $\pm$ 1 S.D. and are derived from 3 repeats.
MCA = Minimum concentration required for a visible agglutination.
NA = none agglutination

4.4 Effect of temperature on the lectin hemagglutination activity and stability

The results of thermal denaturation of the *C. longa* rhizome derived lectin showed that the lectin remains stable when exposed to temperatures below 50 °C for 60 min as determined by the absence of loss of its hemagglutinating activity. Above 50 °C the hemagglutinating activity was gradually lost over time while at 60 °C the hemagglutination activity was reduce to 12.5% and was totally inactivated at 70 °C for more than 30 min (Figure 4.4, 4.5). This is in accord with that reported for the lectin from *Parkia Ja Vanica*, which completely lost hemagglutination activity at 70 °C (Utarabhan and Akkayanont 1995). Correspondingly, the hemagglutinating activity of the *Psophocarpus palustris* lectin rapidly declined when the lectin was heated above 50 °C, was reduced to half at 60 °C and was completely lost at 70 °C (Adenike *et al*., 2005). In contrast, the lectin from the rhizomes of *Smilax glabra* was stable at temperatures up to 50 °C, although above 80 °C very little hemagglutination activity remained suggesting 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). In this work, the thermal stability of the purified *C. longa* rhizomal lectin was found to be stabile between 25 and 50 °C.

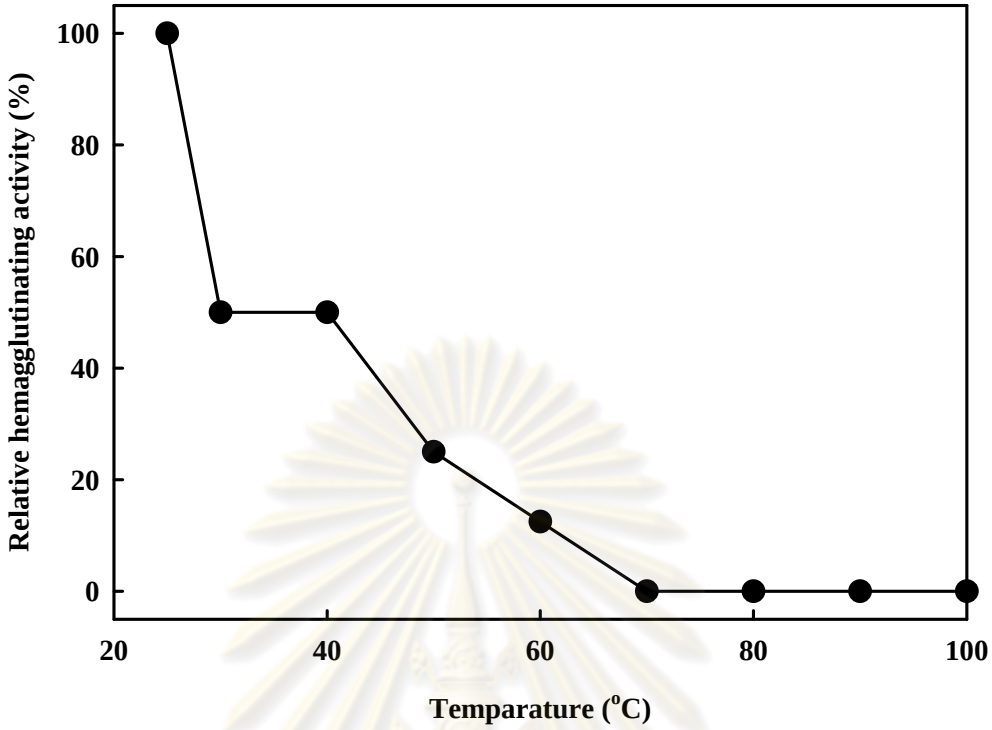

Figure 4.4 Effect of temperature on the agglutinating activity of the purified *C. longa* rhizome lectin towards a rabbit erythrocyte suspension in TBS. The data are shown as the mean $\pm$ 1 S.D. and are derived from three repeats. Full activity (100%) corresponds to a titer of 2^5.

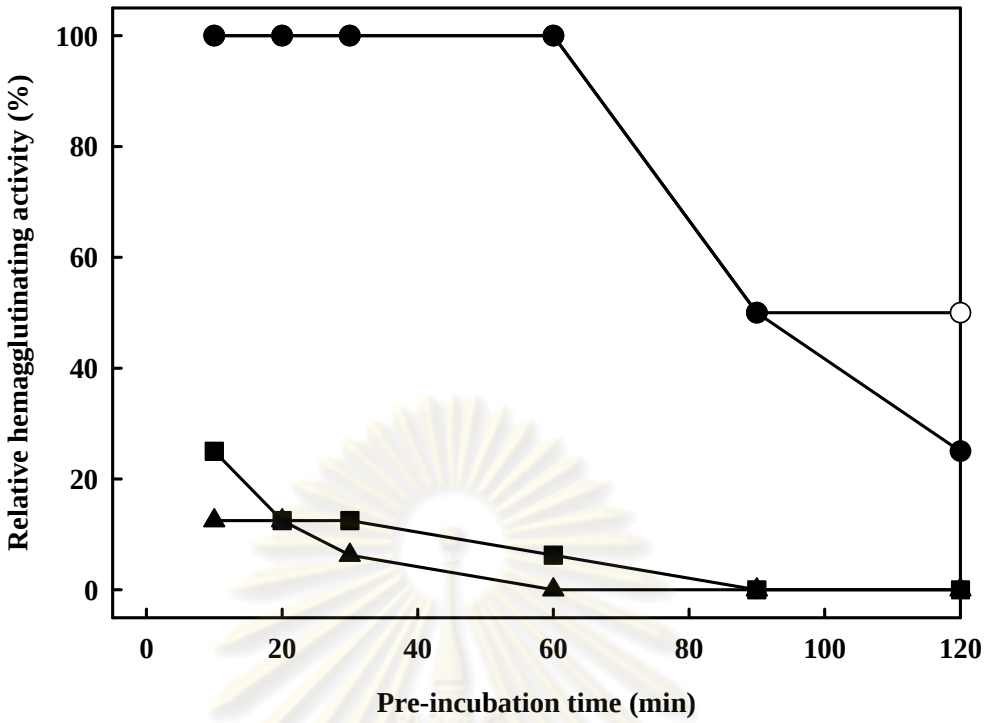

Figure 4.5 Thermostability of the same purified lectin towards a rabbit erythrocyte suspension in TBS at: (○) 40 ^{o}C, (●) 50 ^{o}C, (■) 60 ^{o}C and (▲) 70 ^{o}C. The data are shown as the mean ± 1 S.D. and are derived from three repeats. Full activity (100%) corresponds to a titer of 2^5.

4.5 Effect of pH on the lectin hemagglutination activity

When the purified *C. longa* rhizome lectin activity was tested at different pH values, it was found to be acid tolerant, with the same hemagglutination activity as the control (100%) at all pH values between 2 and 6 (Figure 4.6). In contrast, the lectin rapidly lost its activity at all pH values of 8.0 or higher, decreasing to 25% and 12.5% of the control hemagglutinating activity at pH 8 - 10 and pH 10 - 12, respectively. Interestingly, the hemagglutination activity of the lectin in potassium phosphate buffer at pH 6.0 - 7.0 was almost twofold higher than the control (Figure 4.6). The loss of lectin agglutination activity at alkali pH values seen here with the *C. longa* rhizome lectin has been reported before for other plant lectins, such as the 50% loss of activity at pH 8.5 with the lectin from *Helianthus tuberosus* L. tubers (Suseelan *et al* 2002).

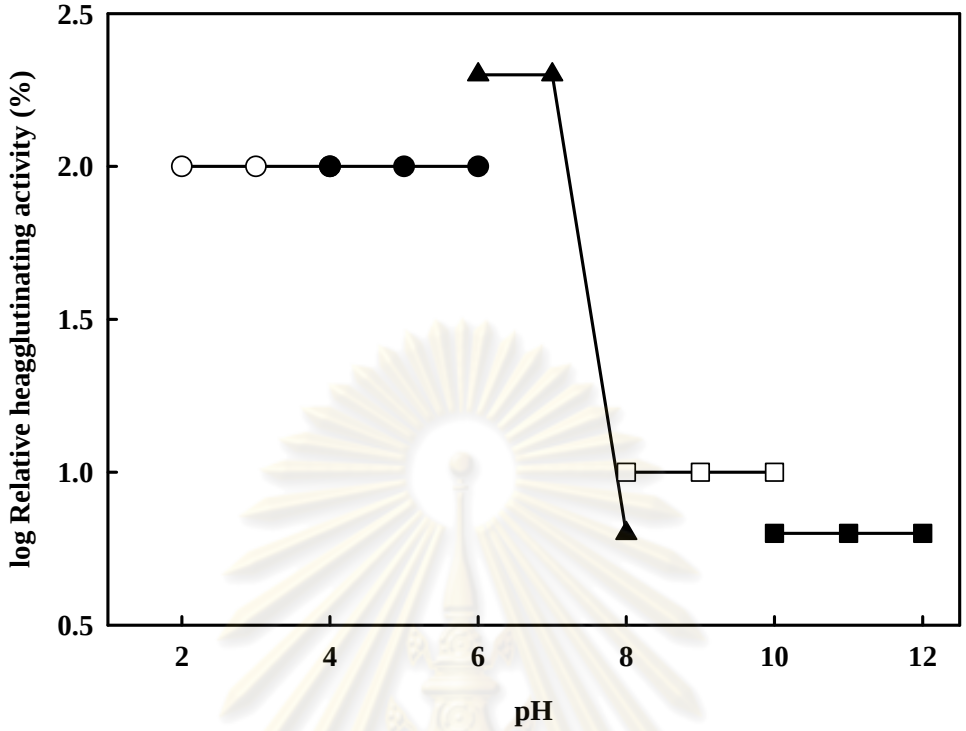

Figure 4.6 The effect of pH on the hemagglutinating activity of *C. longa* rhizome lectin towards rabbit erythrocytes. The data are shown as the mean ± 1 S.D and are derived from three repeats. Full activity (100%) corresponded to a titer of 2^5. The following buffer systems were used: (○) 20 mM glycine-HCl (pH 2.0 - 4.0), (●) 20 mM sodium acetate (pH 4.0 - 6.0), (▲) 20 mM Ppotassium phosphate (pH 6.0 - 8.0), (□) 20 mM Tris-HCl (pH 8.0 -1 0.0) and (■) 20 mM glycine-NaOH (pH 10.0 - 12.0). The data are shown as the mean ± 1 S.D. and are derived from three repeats. Full activity (100%) corresponds to a titer of 2^5.

4.6 Effect of metal ions on the hemagglutination level

Incubating the purified lectin (about 1 mg/ml) with 0, 12.5, 25, 50 and 100 mM of Ca^{2+}, Mn^{2+}, Mg^{2+}, Fe^{3+}, Hg^{2+}, Co^{2+} and EDTA for 24 hours prior to assaying for hemagglutination activity, revealed a *C. longa* rhizome lectin requirement for Ca^{2+} and Mn^{2+}, with both metal cations being required at less than 50 mM to be effective, suggesting they are essential for the stability of the lectin structure and activity (Table 4.3). In contrast, Mg^{2+}, Fe^{3+}, Hg^{2+}, Co^{2+} and EDTA did not support hemagglutinating

activity. This result agrees with the report that the hemagglutination activity of the galactose / N-acetylgalactosamine-specific lectin from *Sophorus juponicus* was completely inhibited by the addition of 0.23 mM EDTA (Iglesias et al., 1982). 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*.

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

The sequence analysis of a partial internal fragment of the purified lectin from *C. longa* rhizomes, obtained by in gel digestion with trypsin and subsequent sequence analysis with LC/MS/MS, was performed. The products of the spectra were obtained and peptide sequences were then determined according to a *de novo* sequencing algorithm to derive a short list of possible sequence candidates. These served as query sequences in a subsequent homology base search against the in-house swiss-prot non-redundant protein database using MS BLAST (Shevchenko *et al*., 2001) to find likely candidates. As a result, the query sequence, SVLPE WVSVG FSATT GINK (m/z of 1993.25) (Figure 4.7), which was similar to those previously isolated for a phytohemagglutinin precursor from *Phaseolus vulgaris* (Q8RVX9) and *P. coccinous* (Q8RVY4) at amino acid residues 218 to 236, suggests that this protein could be a member of this lectin family as well (Figure 4.8).

		5					10					15									Accession Number
***Curcuma longa* lectin**		S	V	L	P	E	W	V	S	V	G	F	S	A	T	T	G	I	N	K		
Phytohemagglutinin precursor (*Phaseolus vulgaris*)	**218**	S	V	L	P	E	W	V	S	V	G	F	S	A	T	T	G	I	N	K	**236**	**Q8RVX9**
Phytohemagglutinin precursor (*Phaseolus coccinous*)	**218**	S	V	L	P	E	W	V	S	V	G	F	S	A	T	T	G	I	N	K	**236**	**Q8RVY4**
Phytohemagglutinin precursor (*Phaseolus costariconsis*)	**218**	S	V	L	P	E	W	V	S	V	G	F	S	A	T	T	G	I	E	K	**236**	**Q5ZF33**
Lectin (*Robinia pseudoacacia*)	**231**	D	V	L	P	E	W	V	R	F	G	F	S	A	T	T	G	I	D	K	**249**	**Q41159**
Lectin (*Glycine max*)	**226**	S	N	L	P	E	W	V	S	V	G	F	S	A	T	T	G	L	H	E	**245**	**B4XQ48**
Lectin (*Glycyrhiza glabra*)	**95**	D	V	L	P	E	F	V	R	I	G	F	S	A	T	T	G	I	S	E	**113**	**Q9ZRN4**
Lectin (*Sophora japonica*)	**245**	T	V	L	P	E	W	V	R	V	G	F	S	A	S	T	G	E	N	V	**263**	**P93537**
Lectin (*Medicago truncatula*)	**221**	K	V	L	P	E	W	V	R	V	G	F	S	A	A	T	G	R	D	F	**239**	**Q43563**

Figure 4.7 Amino acid sequence from the tryptic fragments of the purified *C. longa* lectin. Comparisons are made with other lectins from the lectin family that showed the highest sequence homology in BLAST searches of the NCBI and SwissProt databases. Shaded regions represent regions of identity.

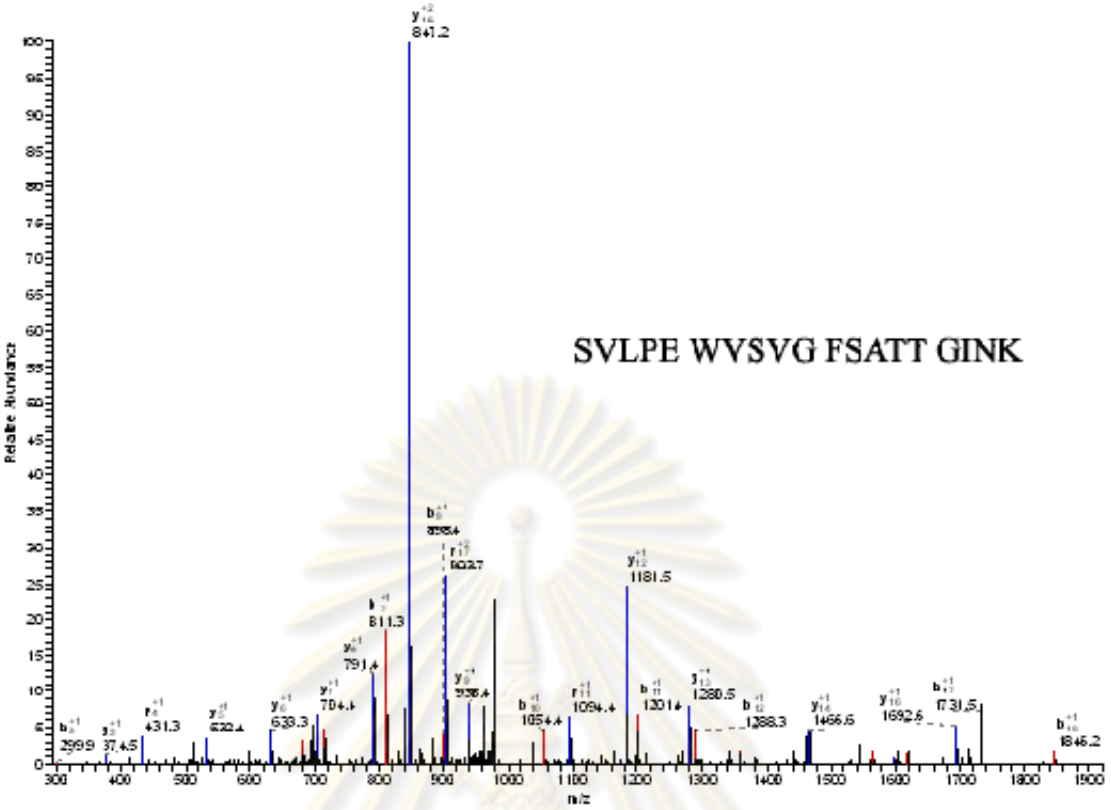

Figure 4.8 LC/MS/MS spectra of the tryptic digest of the purified lectin used to derive the data in (Figure 4.7) above.

4.8 Assay of antifungal activity

The purified *C. longa* lectin at a dose of 47 μg and 94 μg/0.3 cm^2 disc showed antifungal activity against the three tested phytopathogenic fungal species, *Exserohilum turicicum, Fusarium oxysporum* and *Colectrotrichum cassiicola*. While the lectin dose of 47 μg/0.3 cm^2 disc slightly inhibited the growth of these three fungi, that at 94 μg/0.3 cm^2 disc showed a higher and significant degree of antifungal activity on all three isolates (Figure 4.9). This effective lectin dose of around 100 μg/0.3 cm^2 disc is in accord with that reported for the lectin from *Annona muricata* seeds against the growth of *F. oxysporum, F. solani* and *Colletotrichum musae* (Damico *et al.*, 2003), and for the lectin from *Astragalus mongholicus* against *F. oxysporum, Colletorichum* sp. and *D. turcia* (Yan *et al.*, 2005). Other lectins, such as those from potato (Gozia *et al.*, 1995) and red kidney beans (Ye *et al.*, 2001), have also been reported to exhibit antifungal activity. However, novel non-lectin proteins

with antifungal activity in plant rhizomes are also known, such as the 32 kDa protein in ginger rhizomes which exhibits antifungal activity toward *F. oxysporum* at a dose of 32 - 160 μg/0.3 cm^2 disc of ginger rhizome (Wang and Ng 2005)**.**

4.9 Assay of bacterial activity

The antimicrobial activity of *C. longa* lectin, expressed as the minimal inhibitory concentration (MIC), was found to inhibit the growth of all five microbial species tested, the four bacteria, *B. subtilis*, *S. aureus*, *E. coli* and *P. aeruginosa*, plus the yeast *C. albicans*, at MIC values of $\geq$ 0.011, 0.005, 0.092, 0.002 and 0.0046 mg/ml, respectively. These results demonstrate that the *C. longa* rhizome lectin is likely to be at least one of the, if not the, candidate molecule responsible for the antibacterial action observed in rhizome extracts from this plant. An outstanding feature of the antibacterial activity of the isolated lectin is it is somewhat nonselective against this fairly diverse selection of bacteria. The potentially broad effect of the *C. longa* rhizome lectin on the growth inhibition of several diverse bacterial strains, confirms the important interaction between the lectin and all the strains under consideration. From the tested strains, *P. aeruginosa* (lowest MIC) seemed to be most sensitive to the presence of lectin. Previouse studies of the binding of plant lectins to bacterial cell wall peptidoglycans indicate that several lectins of different carbohydrate specificities can recognize most of the components of the bacterial cell wall, such as muramic acid, *N*-acetylglucosamine, *N*-acetylmuramic acid and muramyl dipeptide (Ayouba *et al*., 2006).

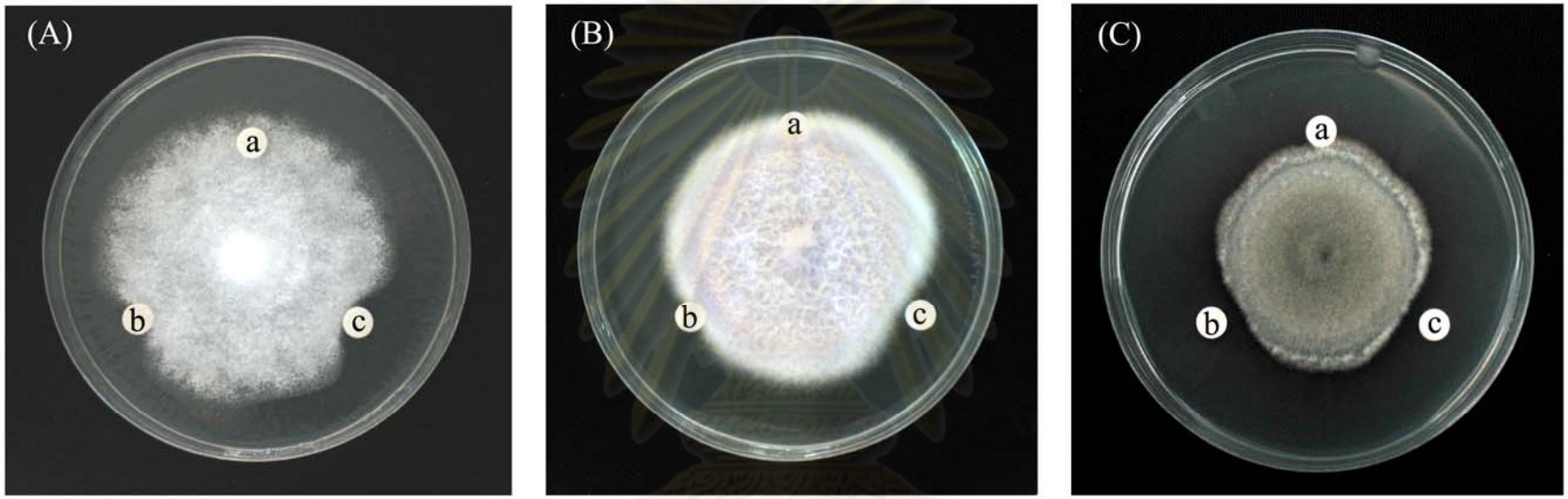

Figure 4.9 The antifungal (colony growth inhibition) effect of the *C. longa* rhizome lectin towards (A) *E. turicicum*, (B) *F. oxysporum* and (C) *C. cassiicola*, showing the (a) negative control (10 µl of 20 mM Tris-HCl buffer pH 7.2), (b) 47 µg/ 0.3 cm^2 disc and (c) 94 µg/ 0.3 cm^2 disc of *C. longa* rhizome lectin.

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). Carbohydrates are digested to monosaccharides which can be absorbed through the intestine. Hence, α-glucosidase inhibitors reduce the impact of carbohydrates on blood sugar. 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 maximal inhibition concentration (IC_{50}) of the purified *C. longa* rhizome lectin was found to be about 8 μg/ml of protein. Therefore, this *C. longa* lectin may be suitable for the same applications as other currently used α-glycosidase inhibitors and may be a new candidate for disease control.

CHAPTER V

CONCLUSION

The purification of lectin from *C.longa* rhizome required single steps that is affinity chromatography by Con A sepharose column. The lectin was obtained with 91.9 fold purification and 41.2% yields evaluate as 0.12 mg protein/g rhizomes. The specific activity was 645.66 x 10^2 HU/mg proteins. The lectin was found to contain 5.2 % carbohydrate content. The lectin had an apparent molecular weight of 17.3 kDa as analyze by SDS-PAGE. The lectin specific B human blood group and it was specific agglutinin 0.010 and 0.020 mg/ml for rat and rabbit erythrocytes respectively. Effect of temperature on lectin activity it was stable below 50^{o}C for 60 min and complete loss activity at 70^{o}C more than 30 min while the pH optimum at 6-7. The activity of lectin was requirement for metal ion Ca^{2+} and Mn^2 at least 50 mM. The internal amino acid sequence of *C. longa* lectin has similarity the sequence of phytohemagglutinin precursor of *Phaseolus vulgaris*, *Phaseolus coccinous*, *Phaseolus costariconsis* and other plants lectin in Fabaceae family. For other biological activity namely α-glucosidase inhibition activity, assay of phytopathogenic antifungal activity, assay of bateria activities by MIC that are result as follow activity of α-glucosidase inhibitor from *C. longa* exhibited very high activities which IC_{50} was 0.0166 mg/ml. The purified lectin at concentration of 47 and 94 µg/disc showed antifungal activity of the *Exserohilum turicicum*, *Fusarium oxysporum*, and *Colectrotrichum cassiicola*. And finally Minimal inhibitory concentration (MIC) of bacterial assay were determined for purified lectin. The lowest to highest MIC value 0.002, 0.005, 0.011, 0.046 and 0.09 mg/ml was obtained for *P. aeruginosa*, *S. aureus*, *B. subtilis*, *C. albican* and *E. Coli* respectively.

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APPENDICES

APPENDIX A

Preparation for non-denaturing polyacrylamide gel electrophoresis (Native-PAGE)

1. Stock solution

2 M Tris-HCl (pH 8.8)

Tris (hydroxymethyl)-amnomethane 24.2 g

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

1M Tris-HCl (pH6.8)

Tris (hydroxymethyl)-aminomethane 12.1 g

Adjustes pH to 6.8 with 1M HCl and adjusted volumn to 100 ml with distilled water.

1% Bromopenol blue (w/v)

Brompphenol blue 100 mg

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

Filtration will remove aggregated dye.

2. Working solution

Solution A (30% (w/v) acrylaminde, 0.8% (w/v) bis-acrylaminde)

Acrylaminde 29.2 g

N,N-methylene-bis-acrylaminde 0.8 g

Adjust volumn to 100 ml with distilled water

Solution B (1.5 M Tris-HCl pH 8.8)

2M Tris-HCl (pH8.8) 75 ml

Distilled water 25 ml

Solution C (0.5M Tris-HCl pH 6.8)

1 M Tris-HCl (pH 6.8)	50 ml
Distilled water	50 ml

10% Ammonium persulfate

Ammonium persulfate	0.5 g
Distilled water	5 ml

Electrophoresis buffer (25 mM Tris, 192 mM glycine)

Tris (hydroxymethyl-aminomethane)	3 g
Glycine	14.4 g
Dissolved in distilled water to 1 liter without pH adjustment	
(Final pH should be 8.8)	

5x sample buffer

(312.5 mM Tris-HCl pH 6.8, 50% glycerol, 0.05% bromophenol blue)

1M Tris-HCl (pH 6.8)	3.1 ml
Glycerol	5 ml
1% Bromophenol blue	0.5 ml
Distilled water	1.4 ml

3. Native-PAGE

7.5% Seperating gel

Solution A	2.5 ml
Solution B	2.5 ml
Distilled water	5 ml
10% Ammonium persulfate	50 µl
TEMED	5 µl

5.0% Stacking gel

Solution A	0.67 ml
Solution C	1 ml
Distilled water	2.3 ml
10% Ammonium persulfate	30 µl
TEMED	5 µl

Coomassie Gel Stain

Coomassie Blue R-250	1 g
Methanol	450 ml
Distilled water	450 ml
Glacial Acetic Acid	100 ml

Coomassie Gel Destain

Methanol	100 ml
Glacial Acetic Acid	100 ml
Distilled water	800 ml

APPENDIX B

Preparation for denaturing polyacrylamide gel electrophoresis

1. Stock solution

2 M Tris-HCl (pH 8.8)

Tris (hydroxymethyl)-amnomethane 24.2 g

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

1M Tris-HCl (pH 6.8)

Tris (hydroxymethyl)-aminomethane 12.1 g

Adjustes pH to 6.8 with 1M HCl and adjusted volumn to 100 ml with distilled water.

10% SDS (w/v)

Sodium dodecyl sulfate (SDS) 10 g

Adjusted volumn to 100 ml with distilled water.

50% Glycerol (w/v)

100% Glycerol 50 ml

Added 50 ml of distilled water

1% Bromopenol blue (w/v)

Bromophenol blue 100 mg

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

Filtration will remove aggregated dye.

2. Working solution

Solution A (30% (w/v) acrylaminde, 0.8% (w/v) bis-acrylaminde)

Acrylaminde	29.2 g
N, N-methylene-bis-acrylaminde	0.8 g
Adjust volumn to 100 ml with distilled water	

Solution B (1.5 M Tris-HCl pH 8.8, 0.4% SDS)

2M Tris-HCl (pH 8.8)	75 ml
10% SDS	4 ml
Distilled water	21 ml

Solution C (0.5M Tris-HCl pH 6.8, 0.4% SDS)

1 M Tris-HCl (pH 6.8)	50 ml
10% SDS	4 ml
Distilled water	46 ml

10% Ammonium persulfate

Ammonium persulfate	0.5 g
Distilled water	5 ml

Electrophoresis buffer (25 mM Tris, 192 mM glycine, 0.1% SDS)

Tris (hydroxymethyl)-aminomethane	3 g
Glycine	14.4 g
SDS	1 g
Dissolved in distilled water to 1 liter without pH adjustment (Final pH should be 8.3)	

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

1M Tris-HCl (pH6.8)	0.6 ml
50% Glycerol	5 ml
10% SDS	2 ml
2-mercaptoethanol	0.5 ml
1% Bromophenol blue	0.5 ml
Distilled water	0.9 ml

3. SDS-PAGE

15% Seperating gel

Solution A	5 ml
Solution B	2.5 ml
Distilled water	2.5 ml
10% Ammonium persulfate	50 µl
TEMED	5 µl

5.0% Stacking gel

Solution A	0.67 ml
Solution C	1 ml
Distilled water	2.3 ml
10% Ammonium persulfate	30 µl
TEMED	5 µl

Coomassie Gel Stain

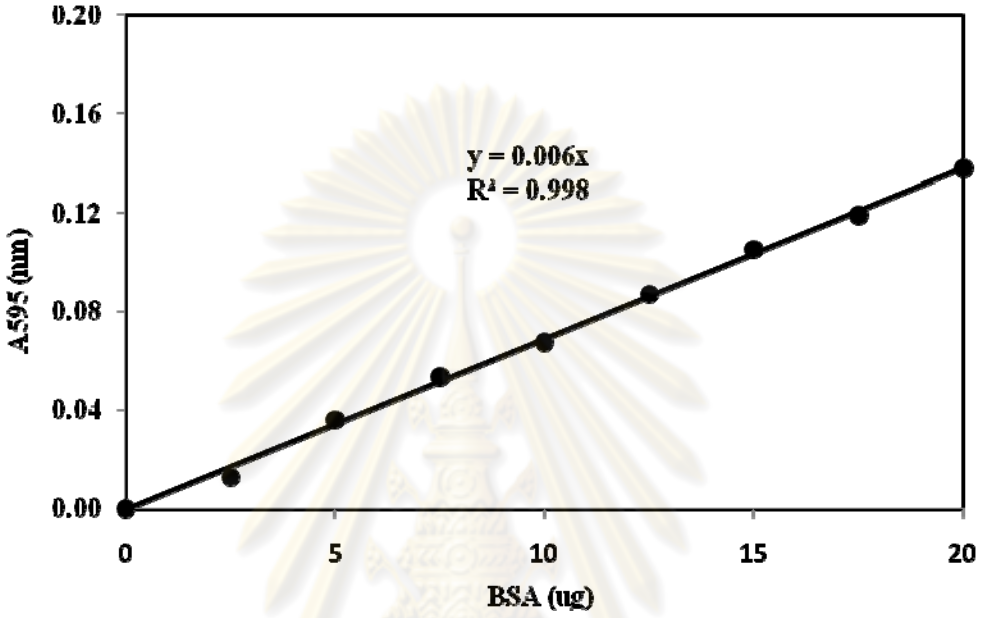
Coomassie Blue R-250	1 g
Methanol	450 ml
Distilled water	450 ml
Glacial Acetic Acid	100 ml

Coomassie Gel Destain

Methanol	100 ml
Glacial Acetic Acid	100 ml
Distilled water	800 ml

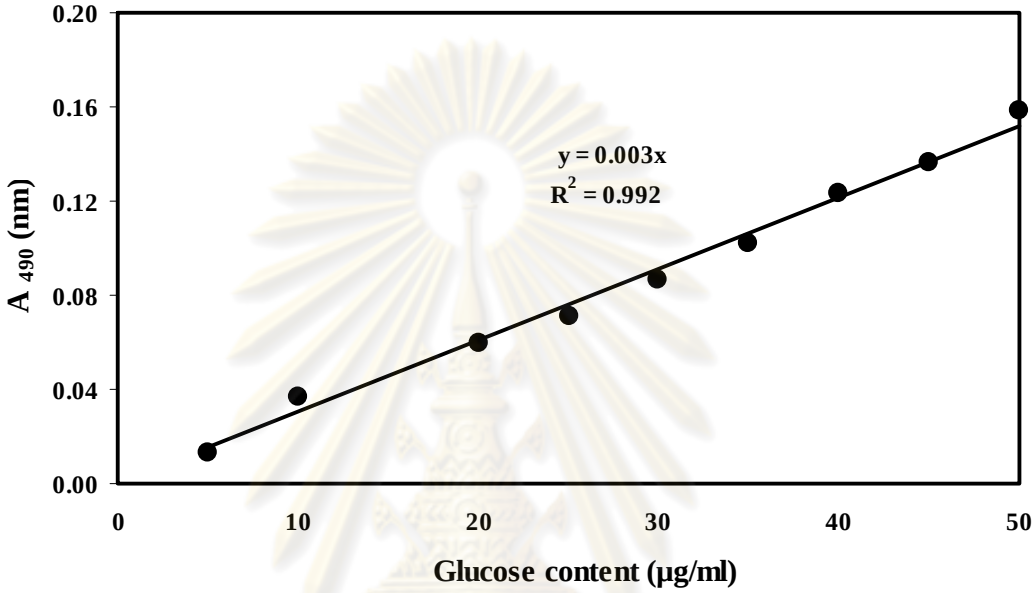
APPENDIX C

Calibration curve for protein determination Bradford method



APPENDIX D

Calibration curve for carbohydrate content determination by phenol sulfuric acid method



APPENDIX E

Culture media

The media were prepared by sterilization in the autoclave at 121^{o}C for 15 minutes.

1. Potato dextrose agar (PDA)

Potato, peeled and diced	200 g
Glucose	20.0 g
Agar	15.0 g
Distilled water	1000 ml

Boil 200 g of peel, dried potato for 1hours in 1000 ml of distilled water. Filter and make up the filtrate to one liter and add the glucose and agar finally sterilize by autoclaving at 121oC for 15 nimutes.

2. Nutrient broth (NB)

Beef extract	3 g
Peptone	5 g
Distilled water	1000 ml

3. Nutrient agar (NA)

Beef extract	3 g
Peptone	5 g
Agar	20 g
Distilled water	1000 ml

4. Yeast Malt broth (YB)

Malt extract	3 g
Yeast extract	3 g
Peptone	5 g
Glucose	10 g
Distilled water	1000 ml

5. Yeast Malt agar

Malt extract	3 g
Yeast extract	3 g
Peptone	5 g
Glucose	10 g
Agar	20 g
Distilled water	1000 ml

APPENDIX F

Abbreviatation for amino acid residues

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

BIOGRAPHY

Miss Pariyaporn Petnual was born on January 26, 1984 in Bangkok, Thailand. She graduated with a Bachelor Degree of Science from the Faculty of Animal Science And Agricultural Technology, Silpakorn University in 2006. She had been studies for a Master Degree of Science in Biotechnology, the Faculty of Science, Chulalongkorn University since 2007.

Academic presentation;

1) Petnual, P., Karnchanatat, A. and Sangvanich, P. Isolate of lectin from rhizomes of *Curcuma longa* L. 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) Petnual, P., Karnchanatat, A. and Sangvanich, P. Isolate of lectin from rhizomes of *Curcuma longa* L. 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.