# การทำให้บริสุทธิ์และลักษณะสมบัติของเลคตินจากเหง้าของขมิ้นดำ *Curcuma amarissima* Roscoe.

นางสาว โนร์ฮามีมี คีรี

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญามหาบัณฑิต
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# PURIFICATION AND CHARACTERIZATION OF LECTIN FROM THE RHIZOMES OF *Curcuma amarissima* ROSCOE.

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**A Thesis Submitted in Partial Fulfillment of the Requirements**
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โนร์ฮามีมี คีรี: การทำให้บริสุทธิ์และลักษณะสมบัติของเลคตินจากเหง้าของขมิ้นดำ *Curcuma amarissima* Roscoe. (PURIFICATION AND CHARACTERIZATION OF LECTIN FROM THE RHIZOMES OF *Curcuma amarissima* Roscoe.) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: ดร. อภิชาติ กาญจนทัต, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: รศ.ดร. พลกฤษณ์ แสงวณิช, 63 หน้า.

ในงานวิจัยนี้ได้ทำการศึกษาลักษณะสมบัติของเลคตินจากเหง้าของขมิ้นดำ *Curcuma amarissima* Roscoe. โดยการนำเหง้าของขมิ้นดำมาสกัดด้วยสารละลายทริสบัฟเฟอร์ที่ค่าความเป็นกรดด่าง 7.2 จากนั้นนำโปรตีนมาทำให้บริสุทธิ์โดยการตกตะกอนด้วยเกลือแอมโมเนียมซัลเฟตอิ่มตัวที่ 80 เปอร์เซนต์ ทำเลคตินให้บริสุทธิ์โดยเทคนิคโครมาโทกราฟีแบบสัมพรรคภาพด้วยคอลัมน์ ConA Sepharose และโครมาโตกราฟีแบบเจลฟิลเตรชันด้วยคอลัมภ์ Superdex G75 ตามลำดับ เมื่อใช้เทคนิคพอลิอะคริลาไมด์เจลอิเล็กโตรฟอเรซิสแบบเสียสภาพ ซึ่งเลคตินบริสุทธิ์ที่ได้มีน้ำหนักโมเลกุลประมาณ 32.4 กิโลดาลตัน โดยจะมีคาร์โบไฮเดรตเป็นองค์ประกอบ 33.21 เปอร์เซนต์ และมีสมบัติในการเกาะกลุ่มกันของเซลล์เม็ดเลือดแดงของหนูแรท กระต่าย และคนในหมู่เลือด A, B, AB และ O โดยมีเสถียรภาพการทำงานที่ทำให้เซลล์เม็ดเลือดแดงเกาะกลุ่มกันที่อุณหภูมิต่ำกว่า 40 องศาเซลเซียส เป็นเวลา 30 นาที ค่ากิจกรรมจะลดลง 50 เปอร์เซนต์ในช่วงอุณหภูมิ 45 จนถึง 85 องศาเซลเซียส และค่ากิจกรรมจะหมดไปเมื่อเพิ่มอุณหภูมิไปจนถึง 95 องศาเซลเซียสเป็นเวลา 30 นาที เลคตินมีกิจกรรมการเกาะกลุ่มกันของเม็ดเลือดแดงสูงที่สุดที่ค่าความเป็นกรด-ด่างเท่ากับ 8 จนถึง 11 กิจกรรมการเกาะกลุ่มกันของเซลล์เม็ดเลือดแดงถูกกระตุ้นได้ด้วยไอออนของแคลเซียม แมกนีเซียม และแมงกานีส และเหล็ก แต่ไอออนของปรอท โคบอลต์ และ อีดีทีเอ สามารถยับยั้งกิจกรรมการเกาะกลุ่มกันของเม็ดเลือดแดงได้ เมื่อวิเคราะห์กรดอะมิโนภายในของเลคตินพบว่าอยู่ในแมนโนส-กลูโคสสเปซิฟิกเลคตินแฟมิลี เลคตินที่ได้สามารถยับยั้งการเจริญเติบโตของเชื้อราโรคพืช *Fusarium oxysporum*, *Exserohilum turcicum* และ *Colletotrichum cassiicola* ได้ ที่ความเข้มข้น 17.5 ถึง 35 ไมโครกรัม และสามารถยับยั้งการเจริญเติบโตของเชื้อจุลินทรีย์สี่ชนิด คือ *Bacillus subtilis, Candida albican*, *Escherichia coli* และ *Staphylococcus aureus* ที่ความเข้มข้นมากกว่าหรือเท่ากับ 0.446, 0.446, 0.223, 0.892 มิลลิกรัมต่อมิลลิลิตร ตามลำดับ เลคตินมีความเป็นพิษต่อเซลล์ มะเร็งเต้านม โดยมีค่าความเข้มข้นของการยับยั้งการเพิ่มจำนวนเซลล์มะเร็งที่ 50 เปอร์เซนต์ เท่ากับ 21.2 ไมโครกรัม และค่าความเข้มข้นการยับยั้งการทำงานของแอลฟา- กลูโคซิเดสที่ 50 เปอร์เซนต์ เท่ากับ 0.073 มิลลิกรัม

สาขาวิชา: เทคโนโลยีชีวภาพ ลายมือชื่อนิสิต .....

ปีการศึกษา: 2552 ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก อภิชาติ กาญจนทัต

ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์ร่วม .....

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NORHAMEEMEE KHEEREE: PURIFICATION AND CHARACTERIZATION OF LECTIN FROM THE RHIZOMES OF *Curcuma amarissima* Roscoe. THESIS ADVISOR: APHICHART KARNCHANATAT, Ph.D., THESIS COADVISOR: ASSOC. PROF. POLKIT SANGVANICH, Ph.D., 63 pp.

A lectin was purified from the rhizomes of *Curcuma amarissima* Roscoe. by aqueous extraction, fractionation with 80% saturated ammonium sulphate, and a combination of affinity and gel chromatography on ConA Sepharose and Superdex G-75, respectively. The molecular mass of the purified lectin was of 32.4 kDa, as estimated by SDS-PAGE. This purified lectin contains 33.21% of carbohydrate and show the specific activity for rabbit and rat erythrocytes but not for mouse, guinea pig, goose, sheep and human blood groups (A, B, AB and O) erythrocytes. This lectin is stable at temperatures below 40 °C, but the hemagglutinating activity was reduced to halved when it was heated up to 45-85 °C and completely lost activity at 95 °C. Surprisingly, the hemagglutinating activity is more stable at 80 °C than at 70 °C, and was rapidly inactivated at 90 °C. It showed a maximum hemagglutination activity within the pH range of 8-11. Metal ions including $Hg^{2+}$, $Co^{2+}$, and EDTA can inhibit its activity whereas $Mg^{2+}$, $Mn^{2+}$, $Fe^{2+}$ and $Ca^{2+}$ were no effect. The deduced amino acid sequence of an internal tryptic peptide sequence of this purified lectin showed sequence similarity (homology) to other members of the leucoagglutinating phytohemagglutinin precursor family, whilst the complete lectin inhibited the *in vitro* growth of three plant pathogenic fungi, *Fusarium oxysporum*, *Exserohilum turicicum*, *Colectrotrichum cassiicola,* at a concentration of 17.5 to 35 µg, and four microbial, *B. subtilis, C. albican*, *E. coli,* and *S. aureus* at concentration ≥ 0.446, 0.446, 0.223, 0.892 mg/ml, respectively. Furthermore, this lectin showed *in vitro* cytotoxicity against the BT474 breast cancer cell line with $IC_{50}$ of ~21.2 µg. Additionaly, $IC_{50}$ for an alpha-glucosidase activity is 0.073 mg/ml protein.

Field of study: Biotechnology Student's signature: Norhameemee Kheeree

Academic year: 2009 Advisor's signature: Aphichart Karnchanatat

Co-advisor's signature: Polkit Sangvanich

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## 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 |

# CHAPTER l

# INTRODUCTION

Lectins are multivalent carbohydrate-binding proteins of non-immune origin that exert biological effects through their ability to specifically bind different carbohydrates structures. (Bies *et al*., 2004; Chandra *et al*., 2006; Rini, 1995; Vijayan and Chandra, 1999) These proteins are ubiquitous in nature occurring in animals, plants and microorganisms (Lis and Sharon, 1998; Mo *et al*., 2000), and present a wide and varied area of applicability including in biotechnological processes and in medical, biological, pharmacological and biochemical research (Bonneil *et al*., 2004; Wong et al., 1998). They have the unique ability to recognize and bind reversibly to specific carbohydrate ligands without any chemical modification, and this distinguishes lectins from other carbohydrate binding proteins and enzymes and makes them invaluable tools in biomedical and glycoconjugate research (Sharon and Lis, 2001). For example, lectins are widespread throughout the plant kingdom, but are of interest as in addition to their relative ease of purification and high yields from plant tissues, they are diverse enough that different families of plants as well as different tissues within the same plant can contain lectins with different molecular properties and carbohydrate binding specificities (Van Damme *et al*., 1998). The functions of plant lectins in nature are extremely diverse and are all based on their ability to recognize and bind the carbohydrate moieties of glycoconjugates, whether in solution or on cells. Because the carbohydrates may be species-, tissue- or cell-specific, certain lectins are capable of discriminating between self and non-self, this being one strategy of innate immunity and a defense mechanism against foreign enemies (Fujita, 2002). The role of lectins in the defense mechanism of plants may have evolved from the ability to lectins to agglutinate and immobilize microorganisms. The supporting evidence for this proposed role in defense against pathogens falls into two main observed categories, namely a) the presence of lectins at potential sites of invasion by infectious agents (Fountain *et al*., 1977) and b) the binding of lectins to various fungi and their ability to inhibit fungal growth and germination (Mirelman *et al*., 1975).

The rhizomes of the Zingiberaceae (ginger) family are widely used in many Asian countries, and their medicinal functions have been broadly discussed and

accepted in many traditional recipes (Sirirugsa, 1998) *Curcuma* is one genus within this family, and it consists of about 80 species in the world. They have been widely cultivated for use as spices (Chaudhary *et al*., 2006; Hermann and Martin, 1991), and food flavoring (Govindarajan, 1980; Khanna, 1999) and coloring(Aggarwal *et al*., 2007; Lin *et al*., 2000), and in traditional medical applications (Baatout *et al*., 2004; Brinkhaus *et al*., 2005; Chearwae *et al*., 2006; Miquel et al., 2002; Wu *et al*., 2000) Also, some species are commonly used in industrial applications. (Kim *et al*., 2006) However, despite the widespread usage of members of this general for medical applications, reports of lectins from *Curcuma* plants are scarce. A mannose-binding lectin from the Zingiberacea member, *C. zedoaria* Rosc., has been identified and found to have sequence similarity to a mannose-binding lectin from the broad-leaved helleborine orchid, *Epipactis helleborine* (L) Crantz (Asparageles: Orchidaceae) (Tipthara *et al*., 2007). In the related *C. aromatica*, six putative proteins from nine bands derived from a rhizome preparation, showed similarity with other lectin sequences(Tipthara et al., 2008) whilst a study on the hemagglutinating activity of *Curcuma* plants found hemagglutinating activity in the species of this study, Khamindum or *C. amarissima* (Tipthara *et al*., 2007).

*C. amarissima*, a member of the ginger, Zingiberaceae, family is used to treat amoebic dysentery, enteritis and as a vermicide. (Larsen, 1996) However, to the best of our knowledge, despite the widespread use of rhizomes from *C. amarissima* in dietary based medical applications there are no report on the lectin from *C. amarissima*. Consequently, the aims of this study were to purify and characterize lectins from the rhizomes of *C. amarissima* and to assay them for bioactivity including antifungal, antimicrobial, antiproliferative, and alpha-glucosidase inhibition activities.

# CHAPTER ll

# LITERATURE REVIEWS

## 2.1 Lectin

### 2.1.1 Definition

Lectin is a protein or glycoprotein that specific recognize for mono and/or oligosaccharide molecule. Lectin one molecule contains at least two specific binding sites. The specificity of a lectin is generally presented in terms of the monosaccharide(s) or simple oligosaccharide that inhibits the lectin-associated reaction. Bond that lectin use to bind with saccharide structure is a weak bond such as hydrogen bond, hydrophobic interaction, and Van Der Waal's force. Lectin has been found in all living organism for example

1) Microorganism lectin; In 1987, had a research was *E. coli* possesse a mannose-specific fimbrial lectin capale of binding to pig ileal epithelial cells (Sharon, 1987), and in 2008 lectin from marine spong (*Halichondria okadai*) that recognizing both Galβ1-3GlcNAc and Galβ1-4GlcNAc was purified. (Kawsar *et al*., 2008)

2) Animal lectin; In 2009, found the lectin from plasma of the shrimp (*Fenneropenaeus chinensis*). It showed a strong affinity for human A/B/O erythrocytes and also has binding activity to some Gram-negative bacteria with cause disease in shrimp and fish. (Sun *et al*., 2008), and in 2009, found an *N*-acetylglucosamine specific lectin that consists of 7.63% sugar from marine bivalve *Macoma birmanica* (Adhya *et al*., 2009)

3) Plant lectin; In 2007 lectin from kidney bean was purified and it contains bioactive compounds capable of inhibiting HIV-1 RT in vitro. (Shi *et al*., 2007) Next year, in 2008 found lectin from *Musa acuminata* (Del Monte banana) that also inhibited proliferation of leukemia cells and hepatoma cells. (Cheung *et al*., 2009)

All of them could be found lectin in plant more than the others. Most common lectin is derived from plant seeds, bark,tubers, pulp, bulbs, and leaves. The part of plant which had a high lectin is a storage part such as, seed, root, and rhizome.

### 2.1.2 History

Lectin, agglutinin, and hemagglutinin are synonym of lectin's names. The first time that Lectin was descript back to 1888 by Stillmark, who study the lectin from the

seeds of castor bean (About Ricin A toxic Ferment from the seeds of *Ricinus communis* L. and some Other Euphorbiaceae Species). He linked the toxicity of castor beans to the occurrence of a hemagglutinaing protein factor. For along time before it was definitely demonstrated that Stillmark's "ricin" was a mixture of a weakly agglutinating protein toxin (still known as ricin) and a nontoxic agglutinin (*Ricinus communis* agglutinin, or RCA). The first evidence for this came from studies by Kabet *et al*. during world war ll. They found by immunochemical methods that the toxic and hemagglutinating properties of "ricin" were due to different substances. Only in 1960 was separation of the two substances achieved by Funatsu. and know that ricin came to the attention of the general public in 1978, following its use as a weapon in the notorious, politically motivated "umbrella murder. The dimensions of the hole, led to the conclusion that ricin was the killing agent, since very few poisons are sufficiently potent to kill a man at such a minute amount. (Van Damme *et al*., 1995) In 1898 Elfstrand introduced for the first time the term "hemagglutinin" as a common name for all plant proteins that cause clumping of cells. (Elfstrand, 1898) The idea that toxicity is an intrinsic property of lectin was abandoned in the beginning of the century after have a report for the first time the present of nontoxic lectin in the legumes *Phaseolus vulgaris* (bean), *Pisum sativum* (pea), *Lens culinaris* (lentil), and *Vicia sativa* (vetch) (Landsteiner and Raubitschek, 1907). Following this work more nontoxicity plant hemagglutinin has been found. Eventually, it became Evident that lectin is widespread in the plant kingdom and that toxicity is the exception rather than the rule.

The next milestone in the history of plant lectin was a term. When have been found that some hemagglutinin exhibit a clear preference toward erythrocytes of a particular human blood group within the ABO system. (Boyd and Reguera, 1949; Renkonen, 1948) The term "lectin" originally introduced to emphasize the selective agglutination behavior of some hemagglutinin, it was later applied to all proteins with agglutinating activity. "Hemagglutinin" is certainly a more appropriate term than lectin because it refers to the capability of a protein to agglutinate erythrocytes but does not take into account that most lectin can also agglutinate other cells. Hence, the term agglutinin should be preferred. In the absence of a clear consensus, the term lectin is actually most commonly used, but agglutinin and hemagglutinin still persist as synonyms.

The current confusion in the terminology of lectin to a great degree is result in the fact that different names have been introduced before the mechanism causing the macroscopically visible agglutination activity was understood in molecular terms. In 1936 already observed that cane sugar inhibited the agglutination activity of Concanavalin A (Con A). (Summer and Howell, 1936) And it was demonstrated in 1952 that the agglutination properties of lectin is base on a specific sugar-binding activity. (Watkins and Morgan, 1952) As soon as lectin was recognized as carbohydrate-binding protein they could be distinguished from other proteins on the basis of a well-defined funchional criterion. For this reason lectin is now considered initially as carbohydrate-binding proteins rather than as (hem) agglutinin.

**2.1.3 Biological activity**

**2.1.3.1 Cell agglutination**

One of lectin properties is agglutinate cells. Lectin will be binding the cell at specific carbohydrate binding sites. The commonly cells was used to determine the lectin agglutinate such as; red blood cells, lymphocyte cells, and bacteria cells. Lectin from the plasma of the shrimp (*Fenneropenaeus chinensis*; FC-L) has a report to agglutinate Gram-negative bacteria which caused disease in shrimp and fish. It recognizes the terminal NeuAc groups in the oligosaccharide chain of glycoconjugates, the acetyl group in the monosaccharide derivatives or LPS on the surface of bacteria. (Sun *et al*., 2008). In addition the lectin from marine red alga (*Gracilaria ornate*) can agglutinate trypsin-treated red blood cells from rabbit and chicken. (Leite *et al*., 2005)

**2.1.3.2 Carbohydrate specificity**

Lectin has a property is specific bind to carbohydrate. The lectin activity was inhibited by specific carbohydrate cause of this carbohydrate will be binding at specific binding site of lectin instead the interesting sample. The report of this property for example, The lectin from rhizomes of *Ophiopogon japonicus* has a carbohydrate specificity for mannose derivative such as, Man-α(1,3:1,6)-mannotriose, Man-α(1,3)-Man, Man-α(1,6)-Man, Man-α(1,2)-Man, Meα-D-man and D-mannose. (Tian *et al*., 2008). In the same year had a report that lectin from *Musa acuminata* (Del Monte banana) was inhibited by glucose, mannose, fructose, glucosamine, and rhamnose. (Cheung *et al*., 2009)

**2.1.3.3 Anti-proliferative activity**

Cause of carbohydrate specific property, so could be applied to use lectin for anti-proliferative activity. The effect of *Arundu donex* lectin (ADL) was determined over a range of 1-50 µg/ml. The ADL showed maximum effect of 62% against ovarian cell line followed by CNS, Liver, Colon, and CNS cell lines, where 59%, 53%, 51% and 45% growth inhibition was observed, respectively, but not inactive for prostate cell line. (Kaur *et al*., 2005). In 2007, had a report of anti-proliferative activity from *Aspidistra elatior* lectin (AEL). The AEL also showed significant *in vitro* anti-proliferative activity towards Breast (66%), Lung (60%) and Liver (56%) of human cancer cell lines but inactive for Prostate, and cervix of human cancer cell lines (Xu *et al*., 2007).

**2.1.3.4 Anti-fungal activity**

In 2007 Sitohy *et al*., had a research of a plant lectin isolated from the Egyptian seeds of *Pisum sativum* can inhibited the growth of fungal consist of *Aspergillus flavus, Trichoderma viride* and *Fusarium oxysporum*. (Sitohy *et al*., 2007). Research of a lectin with antifungal from red cluster pepper (*Capsicum frutescens*) seeds. This lectin was capable of inhibiting the germination of *Aspergillus flavus* and *Fusarium moniliforme* spores and hyphal growth in the two fungi (Ngai and Ng, 2007).

**2.1.3.5 Anti-viral activity**

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

**2.1.3.6 Anti-viral activity**

In 2003, found a research of lectin from *Bufo arenarum* skin. They isolated two β-galactoside-binding lectins. The antimicrobial analysis for each lectin was

performed. At µmolar concentration lectins show strong bacteriostatic activity against Gram negative bacteria, *Escherichia coli* K12 4100, and wild strains of *E. coli* and ,*Proteus morganii* and Gram positive bacteria (*Enterococcus faecalis*). (Alicia Sanchez. *et al*., 2003). In 2008 found to analyzing the antimicrobial properties of a purified lectin from *Eugenia uniflora* L. seeds (EuniSL). The EuniSL demonstrated a remarkable nonselective antibacterial activity. EuniSL strongly inhibited the growth of *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Klebsiella* sp. with a minimum inhibitory concentration (MIC) of 1.5 µg/ml, and moderately inhibited the growth of *Bacillus subtilis*, *Streptococcus* sp. and *E. coli* with a MIC of 16.5 µg/ml. (Oliveira *et al*., 2008)

**2.1.4 Purification technique of lectin**

The lectin was purified from Crude extract of mixer solution, commonly use chromatography technique such as, affinity chromatography, ion exchange chromatography, and gel filtration chromatography. In 2004 had a research that used affinity chromatography to purify the lectin from human serum proteins by Con A sepharose column coupled to two-dimensional gel electrophoresis. The purified sample had 2 fractions before use this technique. (Rodriguez-Pineiro *et al*., 2004). Next year, a lectin from the marine red alga *Gracilaria ornata* (*Gracilariaceae, Rodophyta*); GOL was purified by 2 steps chromatography technique consist of ion exchange chromatography on DEAE-cellulose and affinity chromatography on mucin-Sepharose 4B. The GOL significantly affected the development of *Callosobruchus maculatus* larvae, indicating the possibility of using this lectin in a biotechnological strategy for insect management of stored cowpea seeds. (Leite *et al*., 2005). In 2007 Shi *et al*. study lectin from raw and canned red kidney bean (*Phaseolus vulgaris*). They used gel filtration technique to purify. Use Affi-gel Blue gel sepharose compare to thyroglobulin-Sepharose to purify the lectin from red kidney bean. Found that the lectin from thyroglobulin more purify than Affi-gel Blue gel. (Shi *et al*., 2007)

**2.1.5 Applications of lectin**

**2.1.5.1 Application to clinical microbiology**

In 1936, a using lectin in clinical microbiology began when Summer and Howell (Summer and Howell, 1936) had a report that concanavalin A can agglutinated various *Mycobacterium* spp. The interactions between plant lectin and

microorganisms have been applied for typing of bacteria, fungi, and protozoa. It is useful for characterizing bacterial cell components and for detecting bacteriophage receptors. (Etzler, 1983; Lis and Sharon, 1986; Nicolson, 1974). The unique property of lectin to bind non-covalently to simple sugars and polysaccharides has attracted interest in microbial taxonomy. Lectin has a role in the clinical laboratory identification and taxonomic classification of many microorganisms. Because lectins are generally monoclonal proteins and because they possess a spectrum of specificities and molecular weights, they are substantial tools for diagnostic microbiology applications.

**2.1.5.2 Gram-positive bacteria**

Lectin has been used for investigating virulence factors, surface structures, and identification of gram-positive bacteria. For example; lectin from *Dolichos biflorus* was used to confirmed its specificity for identifying group C streptococci. In another test, its crude extract was coupled to polystyrene particles with a spacer arm to yield an effective lectin-latex reagent that agglutinated group C streptococcal antigens prepared as nitrous acid, autoclave,or enzyme extracts. (Slifkin and Gil, 1984) Group C streptococcal isolates from horses and cattle agglutinated with lectin from *Dolichos biflorus* and *Helix pomatia*. (Schalla *et al*., 1986). Concanavalin A could be precipitated various bacterial polysaccharides, with interacts specifically with bacterial cell walls containing glycosidic residues associated with teichoic acid. Accordingly, bacteria teichoic acids from cell wall containing alpha-glucopyranisyl residues,such as *Lactobacillus plantarum*, *Staphylococcus aureus*, and *Bacillus subtilis*. (Archibald and Coapes, 1971; Doyle *et al*., 1982; Reeder and Ekstedt, 1971). Lectins from soy bean have been used to assay for detecting *B. anthracis*.Cole *et al*. (Cole *et al*., 1984) The use of soybean agglutinin (SBA) to detect very low numbers of buffered suspention of *B. anthracis* vegetative cells and spores has been reported (Graham et al., 1984). The strategy was to bind the cells or spores to polystyrene plates and to detect the bound forms with horseradish peroxidase labeled soybean agglutinin (called the lectinosorbent assay).

**2.1.5.3 Gram-negative bacteria**

The contrast of gram-negative bacteria and gram-positive bacteria is the cell wall of gram-negative bacteria contains to lipid but cell wall of gram-positive bacteria does not have the lipid. In 1968, Doyle *et al*., provided evidence that concanavalin A

reacts with macromolecules that are devoid of terminal glucopyranose or mannopyranose residues. (Doyle *et al*., 1968). Their investigations demonstrated that concanavalin. A precipitates lipopolysaccharide preparations derived from various strains of *E. coli* as well as from *Shigella flexneri* and *Salmonella abortivoequina*. In 1970, other investigators demonstrated that concanavalin A can be used to detect lipopolysaccharides of various Salmonella strains as determined by gel diffusion. (Goldstein and Staub, 1970)

**2.1.5.4 Mycology**

Several investigators have concluded that lectins are useful reagents for the study of fungal cell surfaces and may also be of value as important aids in the classification of fungi. (Barkai-Golan and Sharon, 1978) The major components of fungal cell wall is Chitin, a polymer of β,-(1-4)-*N*-acetyl-D-glucosamine (Barkai-Golan and Sharon, 1978; Ebisu *et al*., 1977). The report of lecin interaction to fungal, such as Fluorescein-conjugated wheat germ agglutinin has been shown to be an effective probe to detect chitin on hypha surfaces. (Barkai-Golan and Sharon, 1978; Galun *et al*., 1976; Galun *et al*., 1981; Mirelman *et al*., 1975; Molano *et al*., 1980; Tkacz and Lampson, 1972; Tracz *et al*., 1971; Tropchin *et al*., 1981) Furthermore, wheat germ agglutinin has been reported to inhibit growth and spore germination of the fungus *Trichoderma viride* (Mirelman *et al*., 1975).

**2.1.5.5 Parasitology**

A large body of data exists on the interaction of lectins with a relatively broad spectrum of parasites ranging from the protozoa through the metazoa. Although concanavalin A was used by many investigators as a lectin probe for these organisms, many other lectins have been shown to be of value in the study of cell surfaces and the identification and differentiation of the parasites. In some instances virulence of parasitic protozoa appears to be related to their surface properties, as revealed by interactions with lectins. Thus, several investigators have deemed important the comparison of surface saccharides of parasites known to differ in their virulence traits. It has been conjectured that the virulence of the trophozoite form of *Entamoeba histolytica* may depend, in part, on its surface properties. Data have been presented indicating that only strains isolated from cases of amoebic dysentery agglutinate with concanavalin A (Martinez-Palomo *et al*., 1973) strains isolated from asymptomatic cases of amoebic dysentery, however, do not agglutinate with this lectin.

**2.1.5.6 Virology**

The unique property of lectins to bind noncovalently to simple sugars and therefore to polysaccharides and glycoconjugates has attracted the interest of virologists. In virology, lectins have been used for detection of viral glycoproteins in purified and infected cells, as well as for viral purification. Lectin studies have revealed information about the structure of viral glycoproteins, structures important in their pathogenicity. A significant contribution of lectin use in virology has been in the development of unique diagnostic methods that yield specific identification of viral agents. Purified influenza virus yields macroscopically visible flocculation when mixed with concanavalin A. (Klenk *et al*., 1984) When influenza virus is treated with a proteolytic enzyme, the glycoprotein spikes of the virus are released. These treated viral particles no longer agglutinate with this lectin, but will flocculate in the presence of N-acetylgalactosamine-associated lectins, such as *Dolichos biflorus* or *Helix pomatia*. Other viruses, including arboviruses, vesicular stomatitis virus, paramyxoviruses, leukoviruses, and hepatitis B virus, also agglutinate with concanavalin A. Concanavalin A was shown to block specifically adsorption of the bacteriophage binding sites of *Bacillus subtilis* possessing alpha-glucosylated teichoic acids in the cell walls associated with teichoic acids. It was suggested that the application of this lectin might be useful as a means to correlated bacteriophage and serologic typing of staphylococci. (Archibald and Coapes, 1972)

**2.1.5.7 Bone marrow transplantation**

Soybean lectin was used to treat human bone marrow. They found that soybean lectin agglutination did little to alter the phenotypic composition or function of T cell. (Sherrie *et al*., 1987) The main application of this lectin is for purging human bone marrow for transplantation. The soybean lectin purging is also used experimentally in bone marrow transplantation of leukemia petients, as an alternative agent to other accepted techniques for T-cell depletion, such as monoclonal antibodies. (Lis and Sharon, 1998)

**2.1.5.8 Blood group determination**

The commonly used lectin for typing erythrocytes that had a report is *Dolichos biflorus*, which when diluted optimally, reacts specifically with A1 erythrocytes. (Issitt and Anstee, 1998) This lectin reacts more consistently with erythrocytes from newborns with A1 gene than do human sources of anti-A1. (Issitt and Anstee, 1998)

Another widely used lectin is *Ulex europaeus*, which is specific for erythrocytes having the H antigen. (Nance, 1986) Freshly prepared *Bandeiraea* (Griffionia) *simplicifolia* lectin will react with both A and B erythrocytes, but when aged it reacts only with group B erythrocytes. (Bird, 1977) A wide variety of other lectins derived from seeds, algae, snails, eels, and clams have been demonstrated to agglutinate respective types of erythrocytes. (Gillboa-Garber *et al*., 1988; Nance, 1986)

**2.1.5.9 Derivative preparation**

Due to their carbohydrate-binding specificity, lectin has proved to be powerful tools for the investigation of the cell surface architecture. (Dietz *et al*., 1988; Doyle *et al*., 1982) The lectin derivative can prepare by labeled the lectin with enzyme (e.g. peroxidase), electron-opague molecule (e.g. ferrilin), fluorescence dyes, or metal particles (e.g. colloidal gold). It has been well known and were employed as histochemical and cytochemical agents for detecting glycoconjugates in tissue sections, on cells and subcellulate organells. In addition lectin binding has been used to demonstrate that membrane receptors for hormones, growth factors, neurotransmitters and toxics, are glycoconjugated, and study intracellular pathways of protein glycosylation.

The rationale for these studies is that they could not only allow the mapping of the cell surface carbohydrates, but also contribute to a better understanding of various surface related biological phenomena, such as cell-cell interactions, immune response morphogenesis drug resistance, or cell attachment. Among the enzymatic markers, horseradish peroxidase (HRP) has been the most widely applied. HRP once bound to lectin, is easily detectable via an osmiophilic raction using 3,3'-diaminobenzidine as the substrate. (Allen *et al*., 1980) However, this technique has a limitation since diffusion of the reaction products away from the original binding sites does not allow an accurate localization of a given carbohydrate. In the case of particulate markers, these include ferritin (Doyle *et al*., 1968), hemocyanin (Galun *et al*., 1976), iron-dextran (Dorai *et al*., 1982), and iron-mannan. (Eaves and Doyle, 1988) More recently, colloidal gold has been introduced in the field of cytochemistry as an alternative to previous particulate markers. (Davidson *et al*., 1982; Ebisu *et al*., 1977) Because gold particles are electron dense, uniform in size, and can be reproducibly and easily prepared, they represent actually most attractive markers for both scanning and transmission electron microscopy. (Boyd, 1963)

#### 2.1.5.10 Mitogenic stimulation

Prior to the advent of monoclonal antibodies to cell surface antigens, lectin was the major tool for studies of the mechanism of cell activation. Mitogenic stimulation by lectin provides an easy means to assess the immunocompetence of patients suffering from diversity of diseases, including AIDS, and to monitor the effects of various immunosuppressive and immunotherapeutic manipulations. (Lis and Sharon, 1998)

#### 2.1.5.11 Cancer diagnosis

Plant lectins have been found the selectively agglutinating property to tumour cells. It has been revealed that neoplastic cells are differing from normal cells at the glycoconjugates on the cell surface. (Kilpatrick, 2000) Such as the report from Carol Jones have been found plant lectins are cytotoxic and can be used to select for mutants of animal cells that exhibit structural changes in cell surface carbohydrates reflecting glycosylation defects. In this report used eight isolated lectin mutants of Chinese hamster ovary (CHO) cells that appear to represent three different phenotype classes. These lectin mutants were much more sensitive to the cytotoxic action of normal rabbit serum (NRS) than were the parental cells. (Carol, 1984)

## 2.2 *Curcuma amarissima* Roscoe.

### 2.2.1 General Background

#### 2.2.1.1 Classification

Family: Zingiberaceae

Genus: *Curcuma*

Species: *Amarissima*

Common manes: Kamin dam, Kamin khom

#### 2.2.1.2 Description

*Curcuma amarissima* Roscoe. is a herb perennial belonging to Zingiberaceae family has a terrestrial pseudostem 1 m. tall. The tuberous or non-tuberous rhizome often with tuber-bearing root has a yellow inside, bluish green middle, and white outside. It is usually growth at a combination of clay. The petiole is a reddish brown long petiole. In addition it has got blade green glabrous leaf. It could be treat an amoebic dysentery, enteritis, and vermicide. (Wu and Larson, 1981)


**Figure 2.1** (A) Physical of *Curcuma amarissima*. (B) *C. amarissima* rhizomes. (C) The inside of *C. amarissima* rhizome.

**2.2.1.3 Literature reviews of Zingeberaceae family**

In 2000 have a research of dichloromethane and methanol extracts of 13 Zingiberaceae species from the *Alpinia, Costus* and *Zingiber* genera were screened for antimicrobial and antioxidant activities. The antimicrobial activity of most of the extracts was antibacterial with only the methanol extract of *Costus discolor* showing very potent antifungal activity against only *Aspergillus ochraceos* and all the extracts showed strong antioxidant activity comparable with or higher that of a-tocopherol. (Habsah *et al*., 2000). In 2005, have a research about the biological activity of extracts extracts of *Curcuma zedoaria* and *Curcuma malabarica* tubers. The biological activity of this study is antibacterial and antifungal activity. They found that the extract of petroleum ether, hexane, chloroform, acetone and ethanol extracts exhibited antibacterial as well as antifungal activity. This study is the first report of the antimicrobial properties of *Curcuma malabarica*. The findings also support the use of *Curcuma zedoaria* tubers in traditional medicine for the treatment of bacterial and fungal infections. (Wilson *et al*., 2005)

In 2008, has a study of β-Elemene, a natural plant drug extracted from *Curcuma wenyujin*, has been used as an antitumor drug for different tumors, including glioblastoma. They had a report that anti-proliferation of glioblastoma cells induced by β-elemene was dependent on p38 MAPK activation. Treatment of glioblastoma

cell lines with β-elemene, led to phosphorylation of p38 MAPK, cell-cycle arrest in G0/G1 phase and inhibition of proliferation of these cells (Yao *et al*., 2008).

# CHAPTER lll

# EXPERIMENTAL

## 3.1 Biological material

The fresh rhizomes of *C. amarissima* were purchased from a local market in Bangkok, Thailand. Human blood was obtained from the blood donation office of 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 five human tumor cell lines, BT474 (breast), CHAGO (lung), HEP-G2 (hepatoma), KATO-3 (gastric) and SW620 (colon), were maintained and obtained from The Institute of Biotechnology and Genetic Engineering, Chulalongkorn University, Bangkok, Thailand.

## 3.2 Chemicals and reagents

ConA Sepharose was purchased from Sigma Chemicals Co. (USA). Methyl-α-D-glucopyranoside and EDTA were purchased from Fluka (Germany). The reagents used for SDS-PAGE were obtained from Plusone Pharmacia Biotech (Sweden), except for the low molecular weight protein calibration kit and Superdex G-75 which were both purchased from Amersham Pharmacia Biotech (UK). Brilliant Blue G, Bovine serum albumin, *p*-nitrophenol, and Alpha-glucosidase, Type l: from bakers yeast were the product of Sigma Chemicals Co. (St. Louis, MO, USA). Potassium hydrogen phosphate ($KH_2PO_4$), disodium hydrogen phosphate ($Na_2H_2PO_4$), ammonium persulphate $(NH_4)_2SO_4$, Calcium chloride ($CaCl_2$), mercury chloride ($HgCl_2$), sodium acetate, sodium hydroxide, sodium chloride (NaCl) , and sulfuric acid were obtained from Merck (Germany). Cobalt chloride, copper sulphate, dimethyl sulfoxide, D-glucose, lactose, maltose and manganese chloride were obtained from Fluka (Switzerland). Tris was purchased from USB (USA). Methanol, ethanol, and acetic acid were purchased from Merck (Germany). Double distilled

water was used in this research was prepared with glass water stills (GFL Gesellschaft fur labortecilk mbH, Germany). All other biochemicals and chemicals used in the investigation were of analytical grade.

**3.3 Apparatus and instruments**

SDS-polyacrylamide gel electrophoresis was run in Hoefer$^{Tm}$ miniVE (minivertical), 8x9 cm gels (Amersham pharmacia biotech, Uppsala, Sweden). The 1.5 ml. siliconized eppendorf and pipette tips were purchased from Axygen sciencetific Inc. and Bioline, U.S.A. Liquid chromatography run on AKTA prime (Amersham pharmacia biotech, wikstroms, Sweden) Micropipette (Pipetteman, Gilson, France) Vortex mixer (Vortex-genie 2, Sciencetific Industries, U.S.A.) pH meter (Denver Instrument U.S.A. system) Sonicate (DHA-1000, Branson, U.S.A.) Orbital Shaker (Kika-Werke GMBH&Co., Germany) Refrigerated centrifuge (Himac CR20B2, HHITACHI, Japan) Water Bath Shaking (Memmert, Germany) Power supply (EPS 3500 XL, Pharmacia, England) Dialysis bag (Rockford, U.S.A.) Aytoclave (Isuzu, Seisakushu Co., Ltd., Japan) Freeze dryer (Laboconco, U.S.A.) Microcentrifuge (Biofuge pico Heraeus, Kendro, Germany) Spectrophotometer (TECAN, Astria).

**3.4 Extraction of lectin from the rhizomes of *C. amarissima***

One kilogram of crushed and washed *C. amarissima* rhizomes were blended in TBS (20 mM Tris-HCl, pH 7.2, plus 150 mM NaCl) at a 1:5 kg/L ratio and then stirred overnight at 4 $^{o}$C and filtered through cloth. The filtrate was clarified by centrifugation at 15,000 x g for 30 min at 4 $^{o}$C, the supernatant harvested and the protein precipitated by the addition of ammonium sulfate to 80% saturation on ice and centrifuged as above. The supernatant was discarded and the pelleted proteins were dissolved in TBS, dialyzed against excess water and then freeze dried.

**3.5 Purification of lectin from the rhizomes of *C. amarissima***

**3.5.1 Affinity chromatography**

The freeze dried crude was dissolved in deionized water and centrifuged at 10,000 x g for 10 min. The supernatant was loaded on ConA Sepharose column (1.6 x 20 cm). The unbound was eluted with equilibrating buffer (20 mM Tris buffer saline pH 7.2) and then the bound proteins were eluted with 0.5 M methyl-α-D-glucopyranoside in 20 mM Tris buffer saline. The protein absorption was monitored at 280 nm.

### 3.5.2 Gel filtration chromatography

The bound samples that pass ConA Sepharose Column was load on Superdex G75 Column (16 x 60).And eluted with 20 mM Tris-HCl buffer saline pH 7.2. The protein was monitored at 280 nm.

### 3.6 Protein determination and carbohydrate content

The protein concentration was determined by Bradford assay. (Bradford, 1976) For the standard curve, BSA (bovine serum albumin) was used protein standard solution. Using the microplate for this studied. The samples were used 10 µl into each well then added 100 µl of Bradford working solution. Suddenly the result was determined by microplate reader at 595 nm. In this study used this technique to find carbohydrate content in the lectin sample. The procedure of Dubois (Dubois et al., 1956) was used in this study by use glucose as a standard.

### 3.7 Hemagglutination assay

Serial two fold dilutions of purified protein in 20 mM Tris-HCl buffered saline (50 µl) were incubated with 50 µl of rabbit erythrocyte suspension in U-shaped microtiter plates and the agglutination was stored at room temperature for 1 h. The hemagglutination unit (HU) was expressed as the reciprocal of the highest lectin. Dilution showing detectable visible erythrocyte agglutination and the specific activity was calculated as HU/mg protein. Hemagglutination activity was assayed against rabbit erythrocytes (2-4% rabbit erythrocyte suspension).

### 3.8 Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

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

**3.9 Effect of temperature on lectin hemagglutinating activity and thermo stability**

The effect of temperature on lectin hemagglutinating activity was determined by incubating lectin samples (pH 7.2) at various temperatures (30, 40, 50, 60, 70, 80, 90 and 99 $^{o}$ C) for 30 min. Thermo stability was investigated by incubating lectin samples at 60, 70, 80, and 90 $^{o}$ C in the same buffer without substrate. Samples were removed at fixed time intervals. The residual hemagglutinating activity was assayed after adjusting the temperature to 4 $^{o}$ C. At least three replicates were done for each test to confirm the results.

**3.10 pH-dependence of agglutination activity**

The effect of pH stability on *Curcuma amarissima* lectin was determined by incubating this lectin in various pH ranging from pH 2-12. The buffers were used as follows: 20 mM glycine-HCl pH 2-4, 20 mM sodium acetate pH 4-6, 20 mM potassium phosphate pH 6-8, mM Tris-HCl pH 8-10, 20 mMglycine NaOH pH 10-12. The residual hemagglutinating activity was assayed after adjusting the mixture to pH 7.2

**3.11 Effect of metal ion**

The purified lectin (1 mg/ml) was incubated for 10 h with one of $Ca^{2+}$, $Co^{2+}$, EDTA, $Hg^{2+}$, $Mg^{2+}$, $Mn^{2+}$ and $Fe^{2+}$ at various 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 replicates for each assay.

**3.12 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.

**3.13 Anti-proliferative activity**

Lectin binding in human tumor cell lines has been investigated. The bioassay for in vitro antiproliferative activity toward five cell lines comprising of BT474 (breast), CHAGO (lung), HEP-G2 (hepatoma), KATO-3 (gastric) and SW620 (colon), these cells were trypsinized before seeding at a density of $1\times10^4$ cells/µl in 96 well plates for 24 h, at 37$^o$C in an atmosphere of 5% $CO_2$. Then serial concentrations of the purified lectin were added before further incubation for 72 h. MTT (3-[5, 5-dimethylthyazol-2-yl-2, 5-diphenyltetra zolium bromide) solution (5 mg/ml) was then added following by further incubation for 4 h. Absorbance at 540 nm was measured using microtiter reader after incubation with DMSO (dimethyl sulfoxide; 150 µl/well) for 30 min. Reagents and controls were included with the absence of cells or the crude extract, respectively.

**3.14 Anti-fungal activity**

Antifungal bioassays, using the *E. turicicum*, *F. oxysporum* and *C. cassiicola* plant pathogenic isolates, were performed on 90 x 15 mm petri plates containing 10 ml of standard potato dextrose agar (PDA). After the mycelial colony had developed, sterile blank filter paper disks (0.625 cm in diameter) were placed 1 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.

**3.15 Anti-microbial activity**

Five pathogenic species, namely the four bacterial species, *P. auroginosa, B. subtilis, E. coli* and *S. aureus*, plus the yeast, *C. albicans*, were used to assess the antimicrobial activity of the purified lectin from the rhizomes of *C. longa*. The inoculum of each microorganism was prepared from a six hour old broth culture, and suspensions were adjusted to a McFarland standard turbidity number 0.5. The purified lectin (50 µl) was diluted in serial two fold dilutions in 96-well microtiter plate with

50 μl of broth to which 50 μl of the test microorganism suspension was added in each well, giving a final volume in each well of 150 μl. Broth media alone was used as a negative control. Ampicillin and penicillin (1 mg/ml) were used separately as positive controls to determine the sensitivity of the tested solutions. The plate was covered with a sterile plate sealer and incubated for 24 hours at 37 °C for the four bacterial isolates or at 30 °C for the yeast, *C. albican*s. Thereafter, the absorbance of the mixed suspension at 600 nm was used to indicate the level of growth of the test organism relative to the negative (and positive) control.

**3.16 Alpha-glucosidase inhibition assay**

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 $^{o}$C for 30 min and then 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, where 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 lV

# RESULT AND DISCUSSION

## 4.1 Purification of lectin from *C. amarissima* rhizomes

The present study describes the purification and characterization of a lectin from the rhizomes of *C. amarissima*. Since the crude extract from the rhizomes of *C. amarissima* contained a lot of soluble components, including carbohydrates and other proteins, ammonium sulfate precipitation was applied as the first step of purification. The lectins were found to precipitate with 80% saturation ammonium sulfate and so this initial stage was used prior to purification by affinity and then gel filtration chromatography (Table 1). The initial 80% saturation ammonium sulfate precipitation resulted in an Affinity chromatography with a ConA Sepharose column resulted in two factions, an un-bound fraction that eluted in the TBS wash through and did not show any detectable hemagglutinating activity, and a bound fraction that eluted in the presence of 0.5 M methyl α-D-glucopyranoside and had hemagglutinating activity with a specific activity of 2.21 x $10^3$ HU/mg protein (Figure 4.1).

Affinity chromatography presents advantages in relation to other conventional methods due to its specificity and thus allows a reduced number of steps and gives high yields and purity (Goldenberg, 1989). As such it is widely used in the purification of 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 ConA Sepharose based affinity chromatography column. (Absar *et al*., 2005; Richard *et al*., 1990) However, in some contrast, there have been reports that affinity chromatography could not be applied successfully for the purification of some lectins. For instance, the isolation of *Dolichose biflorus* lectin with *N*-acetyl-galactosamine (NAG) immobilized to Sepharose was not successful and was caused by the substitution of the binding site at the C-6 hydroxyl of carbohydrate in the matrix. Rather, these lectins were resolved by affinity electrophoresis, a combination of affinity and conventional chromatography. (Borrebaeck and Etzler 1980) Another example is the lectin from ground elder (*Aegopodium podagraria*) rhizomes which also could not be purified by Gal-NAG-Sepharose, but by an affinity chromatography of erythrocyte membrane protein

immobilized on cross-linked agarose. (Peumans *et al*., 1985) The recovered bound fraction with hemagglutinating activity was dialyzed and concentrated and then resolved by gel filtration chromatography with a Superdex G-75 column, resulting in fractionation into three distinct peaks, only the last one of which showed any hemagglutinating activity and so was harvested. The final homogeneous lectin preparation obtained was purified by 360.09 fold with 123.20 % recovery and had a specific activity of 32.41 x $10^3$ HU/mg proteins (Figure 4.2). The purification details of this lectin are summarized in Table 4.1. *C. amarissima* lectin contains 33.2% sugar. This lectin had more sugar contain than Chinese evergreen chinkapin lectin (5.8%) (Wong *et al*., 2008) and *Arundu donex* lectin (2.1%). (Kaur *et al*., 2005)

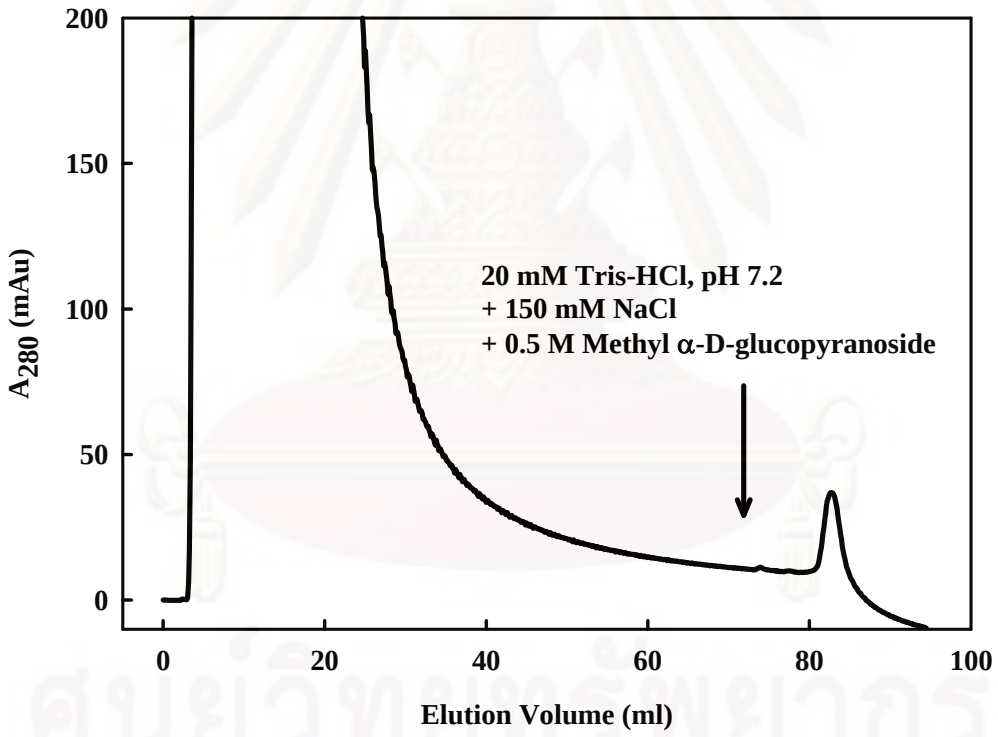


**Figure 4.1** Affinity chromatogram of *C. amarissima* lectin on a ConA Sepharose column equilibrated and then washed with TBS. Lectin was then eluted with TBS containing 0.2 M Methyl α-D-glucopyranoside as described in the experimental section.

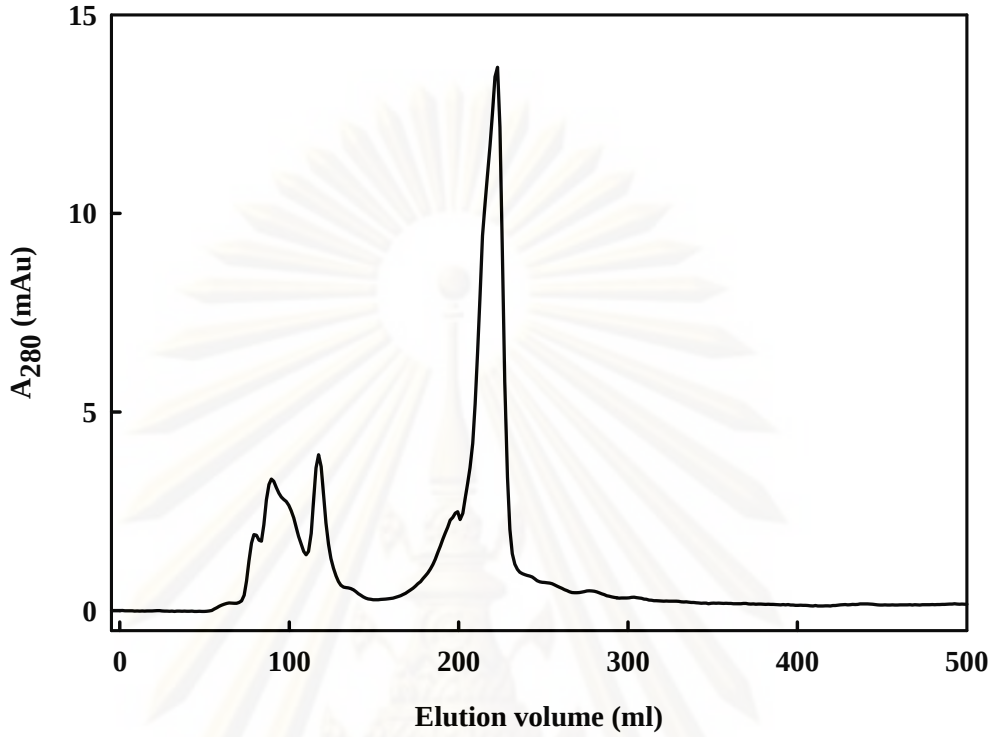


**Figure 4.2** Elution profile of purified *C. amarissima* lectin on a Superdex G-75 column.

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

| **Purification step** | **Total protein (mg)[a]** | **Total activity (HU)[b]** | **Specific activity (HU/mg)[c]** | **Yield (%)** | **Purification (fold)[d]** |
|---|---|---|---|---|---|
| Crude extract | 2152.98 | $1.94 \times 10^5$ | 90.29 | 100 | 1 |
| 80% $(NH_4)_2SO_4$ precipitation | 54.43 | $1.24 \times 10^5$ | $2.28 \times 10^3$ | 64 | 25.32 |
| Con A Sepharose (bound fraction) | 47.76 | $1.06 \times 10^5$ | $2.21 \times 10^3$ | 54.42 | 24.53 |
| Superdex G75 (last peak) | 7.37 | $2.39 \times 10^5$ | $32.41 \times 10^3$ | 122.86 | 358.92 |

[a] Crude protein extract from 200 g 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

Discontinuous SDS-PAGE has been shown to be a relatively sensitive technique for lectin separation (Goldenberg, 1989), and here, in the presence of 2-merceptoethanol under reducing conditions, revealed a single strong band lectin corresponding to an apparent molecular weight of 34.2 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, or at least if a multimeric one that dissociates into subunits under these conditions then this subunit has hemagglutination activity alone. The apparent size of ~34.2 kDa is in agreement with the previously published sizes of the other plant lectins which ranged from 30 to 35 kDa. (Franz *et al*., 1981; Kamemura *et al*., 1996; Van Damme *et al*., 1995).

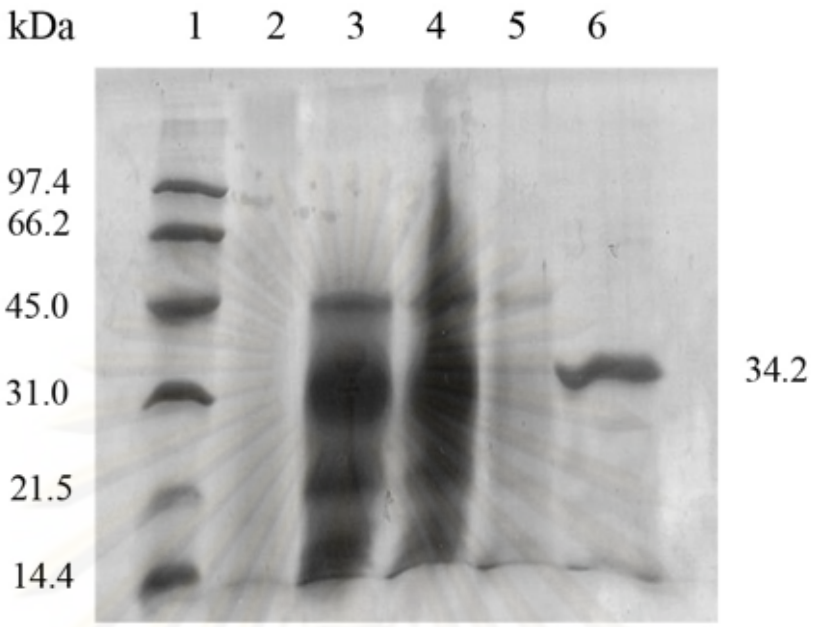


**Figure 4.3** Reducing SDS-PAGE analysis of *C. amarissima* lectin. Lane 1, molecular weight standards; Lane 2, the crude extract (homogenate); Lane 3, the 80% ammonium sulphate precipitated and kept fraction; Lane 4, the non bound ConA sepahrose fraction discarded; Lane 5, the bound fraction kept; and Lane 6, the peak obtained hemagglutinating activity from the gel filtration**.**

**4.3 Assay for hemagglutinating activity**

The carbohydrates specificities of many lectins have been grouped by the ability of monosaccharides or their glycosides to inhibit the lectin-induced hemagglutination (Goldstein and Poretz 1986). However, lectins of the same apparent monosaccharide specificity were found to demonstrate different reactivity's towards different oligosaccharide chains, and differential affinities to animal cells and glycoproteins, which implies that they have their own binding specificity determinants that extend beyond the monosaccharide unit (Gallagher 1984). The lectin from *C. amarissima* rhizomes purified here showed the strongest affinity of all the tested animal erythrocytes to rabbits followed by human blood groups A and AB, then to human blood groups B and O and finally to rat erythrocytes, suggesting that *C. amarissima* lectin recognizes the surface of erythrocyte membranes (Table 4.2). In contrast, no detectable hemagglutination of *C. amarissima* lectin with goose, guinea pig, mouse, and sheep was observed. Several lectins have been reported previously to

demonstrate a preference in agglutinating one more types of human blood groups or animal erythrocytes, such as the lectins from *Pisum sativum* (Sitohy, Mahmoud, Doheim, Mahmoud, & Badr, Haitham, 2007) found to be non-specific for erythrocyte from human erythrocyte group and indiscriminately agglutinate rat and the rabbit erythrocytes. In the case of *Pisum sativum* lectin could agglutinated 3 animal erythrocytes were rabbit, rat, and chicken erythrocytes. In addition *Acorus* lectins agglutinated rabbit, rat and guinea pig erythrocytes. Both *Acorus calamus* (ACL) and *Acorus gramineus* (AGL) also reacted with RBCs from sheep, goat and human ABO blood groups after neuraminidase treatment. (Bains, Jagmohan Singh et al., 2005) Cause of a difference hemagglutinating activity of various erythrocytes was sugar at surface of each erythrocyte.

**Table 4.2** Hemagglutinating activity of the purified lectin from the rhizomes of *C. amarissima* against human and animal erythrocytes.

| Erythrocyte source | Agglutination (titer)[a] |
|---|---|
| Mouse | 0 |
| Rat | $2^2$ |
| Guinea pig | 0 |
| Goose | 0 |
| Sheep | 0 |
| Rabbit | $2^6$ |
| Human Type A | $2^4$ |
| Human Type B | $2^3$ |
| Human Type O | $2^3$ |
| Human Type AB | $2^4$ |

[a]Titer is defined as the reciprocal of the end point dilution causing detectable agglutination of erythrocytes. The concentration of *C. amarissima* lectin used in this assays was 0.045 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.

**4.4 Effect of temperature on the lectin hemagglutination activity and stability**

The *C. amarissima* lectin was stable at temperatures below 40 °C. The hemagglutinating activity decreased to half when it was heated to 45-85 °C. Moreover, its activity completely lost at 95 °C. (Figure 4.4). In the case of a mannose/glucose-specific lectin from Chinese evergreen chinkapin (*Castanopsis chinensis*; CCL), Its activity was stable up to 60 °C for 30 min. Above this temperature, the activity underwent a decline. (Wong *et al*., 2008) The CCL activity is more stable than *C. amarissima* lectin.

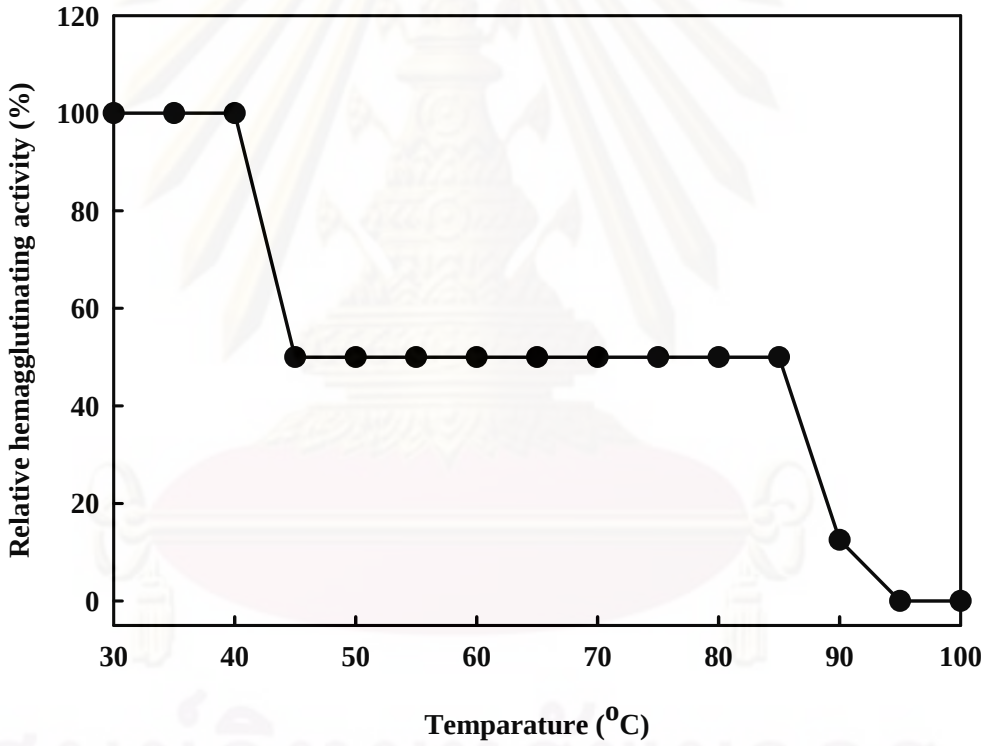


**Figure 4.4** Effect of temperature on the agglutinating activity of the purified *C. amarissima* lectin towards a rabbit erythrocyte suspension in TBS. The data are shown as the mean ± 1 S.D and are derived from three repeats. Full activity (100%) corresponds to a titer of $2^4$.

The thermal stability at various exposure times assay, found only 60 °C 10 min that had100% hemagglutinating activity, but that the activity decreased as the

duration of the exposure increased (Figure 4.5). The thermal stability observed for this *C. amarissima* lectin is comparable to that already reported for some other thermostable lectins treated under similar conditions. (Konozy *et al*., 2003; Oliveira *et al*., 2002). Indeed, the only thermophilic lectin isolated to date is from *Momordica charantia*, which has a maximal activity at 55 °C. (Toyama *et al*., 2008).

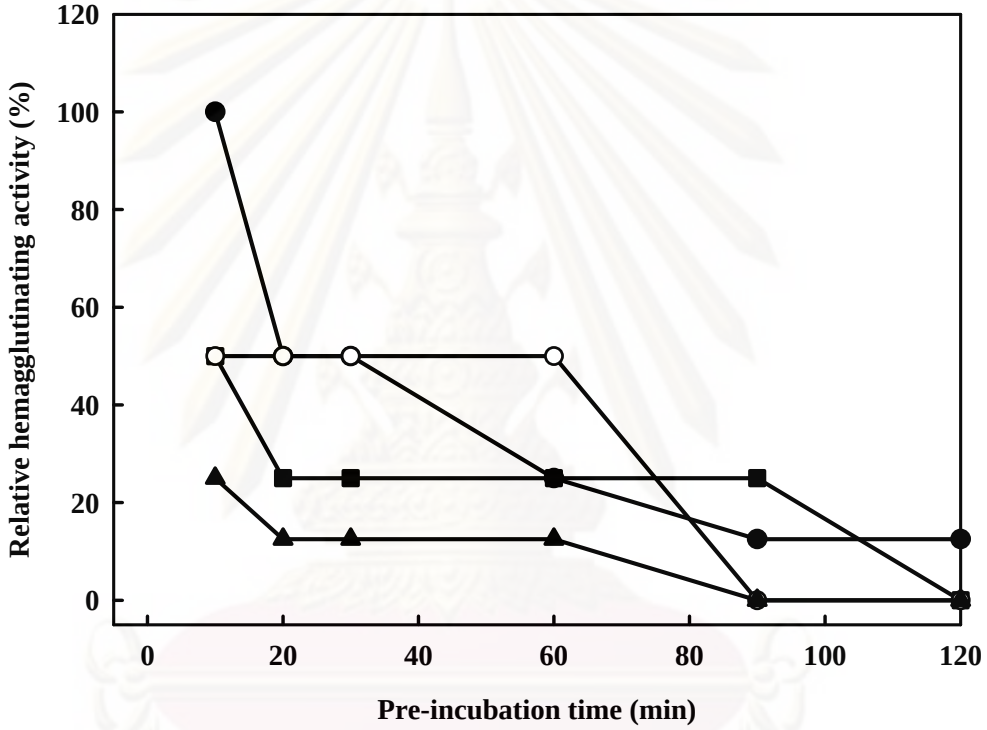


**Figure 4.5** Thermostability of the same purified lectin towards a rabbit erythrocyte suspension in TBS at: (○) 40 $^{o}$C; (●) 50 $^{o}$C; (■) 60 $^{o}$C; and (▲) 70 $^{o}$C. For both panels the data are shown as the mean $\pm$ 1 S.D and are derived from three repeats. Full activity (100%) corresponds to a titer of $2^4$.

**4.5 pH-dependence of agglutination activity**

The pH sensitivity profile of the purified *C. amarissima* lectin is summarized in Figure 4.6, where it can be seen that this lectin exhibited a broad pH optima between pH 8.0-11.0, with a 50% loss of agglutination activity at one pH unit either side, that is at pH 12 or 7. This is in contrast to the other reported lectins, which are

stable over a broader pH range such as the lectins from *Koelreuteria paniculata* seeds, *Talisia esculenta* and *Sphenostyles stenocarpa* which were stable over a pH range of 5-9, 3-9 and 2-10, respectively. (Freire *et al*., 2002; Macedo *et al*., 2003; Machuka. *et al*., 1999) The hemagglutinating ability of the purified *C. amarissima* lectin reported here was totally inactive at a pH of 4 or less, which might be due to the β and α subunits of the lectin dissociating at an acidic condition. (Schwarz *et al*., Regardless the optimum activity of this lectin was at a basic pH (8-11), although higher pH levels induced denaturation.

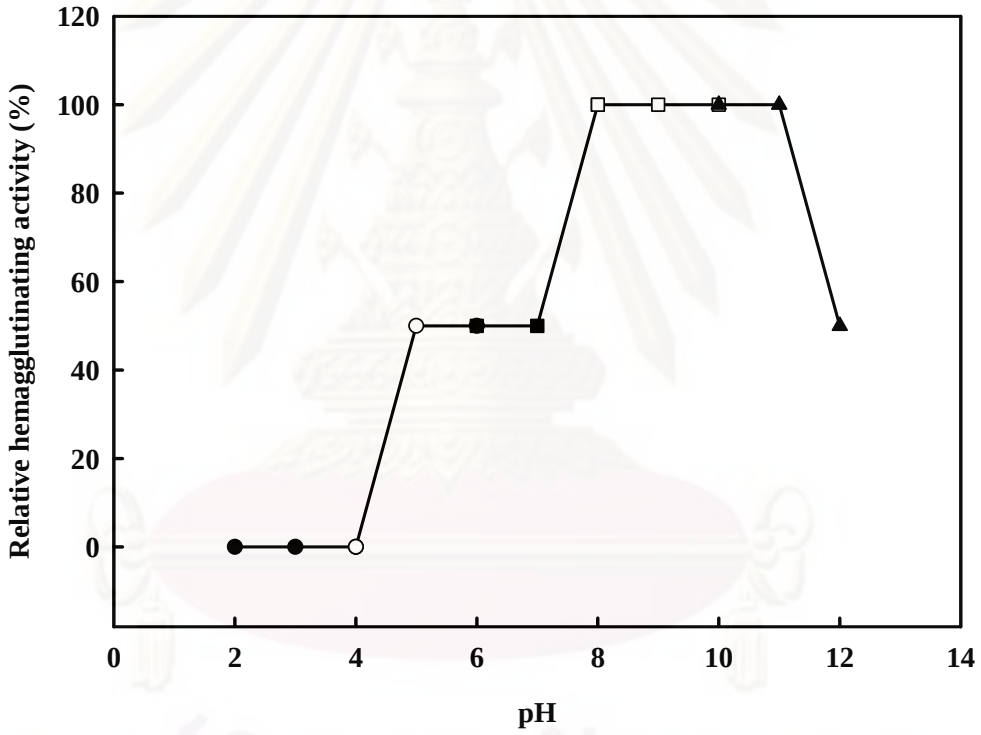


**Figure 4.6** Effect of pH pretreatment on the agglutinating activity of the purified *C. amarissima* lectin towards rabbit erythrocytes in TBS. Pretreatment was with (●) 20 mM glycine-HCl (pH 2-4), (○) 20 mM sodium acetate (pH 4-6), (□) 20 mM potassium phosphate (pH 6-8), (■) 20 mM Tris-HCl (pH 8-10) and (▲) 20 mM glycine-NaOH (pH 10-12). Data are shown as the mean $\pm$ 1 S.D and are derived from three repeats. Full activity (100%) corresponds to a titer of $2^5$.

**4.6 Effect of metal ions on the hemagglutination level**

The effect of divalent cations on *C. amarissima* lectins was evaluated with six different divalent metal ions. $Ca^{2+}$, $Mg^{2+}$, and $Mn^{2+}$ were found to satisfy the requirements for hemagglutination activity, whereas $Co^{2+}$ and $Hg^{2+}$ were not able to support agglutination and $Fe^{2+}$ was only able to support it at high concentrations (Table 4.3). Many lectins have been reported to be metalloproteins and these metal cation cofactors are required for such activities as hemagglutination and indeed the requirement for divalent metal ions like $Ca^{2+}$, $Mg^{2+}$ and $Mn^{2+}$ is a general physico-chemical property of most legume lectins (Goldstein and Poretz 1986; Sharon and Lis 1990) suggesting that they are essential for the hemagglutination activity. For example, the lectin from seed coagulant *Moringa oleifera* required 5mM of $Mg^{2+}$, $Ca^{2+}$ and $K^{+}$ (Santos, et al., 2009) and lectin from *Salvia bogotensis* seed. The lectin's activity therefore depended upon $Ca^{2+}$ and $Mn^{2+}$ being bound to native protein as well as on intact disulfide bridges. (Vega *et al*., 2006) But in contract to lectin from Chinese evergreen chinkapin that no effect on the hemagglutinating activity for $Na^{+}$, $K^{+}$, $Ca^{2+}$, $Mg^{2+}$, $Mn^{2+}$, $Cu^{2+}$ and $Fe^{3+}$ ions at various concentrations (100, 50, 25, 12.5, 6.25, 3.125, and 1.56 mM). (Wong *et al*., 2008)

**Table 4.3** Hemagglutinating activity of the purified lectin from the rhizomes of *C. amarissima* against human and animal erythrocytes.

| Concentration of divalent metal cations (mM) | $CaCl_2$ | $MgCl_2$ | $MnCl_2$ | $FeCl_2$ | $CoCl_2$ | $HgCl_2$ | EDTA |
|---|---|---|---|---|---|---|---|
| 25 | + | + | + | + | - | - | - |
| 50 | + | + | + | - | - | - | - |
| 100 | + | + | + | - | - | - | - |
| 200 | - | - | - | - | - | - | - |

+ hemagglutinating activity

- no hemagglutinating activity

**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. amarissima* rhizomes obtained by in gel digestion with trypsin and subsequent sequence analysis with LC-MS/MS, revealed a peptide fragment with the likely sequence **GNVET NDVLS WSFAS KL** (Figure 4.7). Comparisons to all protein sequences in the SwissProt database using BLASTP searching identified this fragment as a likely homolog of parts of a lectin precursor from the common bean, *Phaseolus vulgeris* L. (Fabales: Fabaceae) (Hoffman and Donaldson1985). The high degree of internal amino acid sequence identity between the peptide fragment of this lectin, from *C. amarissima* rhizomes, with those of other members of the leucoagglutinating phytohemagglutinin precursor family (Figure 4.8) suggests that this protein could be a member of this lectin family as well.

| | | 1 | | | | 5 | | | | | 10 | | | | | 15 | | | Accession Number |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *Curcuma amarissima* lectin | | G | N | V | E | T | N | D | V | L | S | W | S | F | A | S | K | | |
| Leucoagglutinating phytohemagglutinin precursor (*Phaseolus vulgaris*) | 236 | G | N | V | E | T | N | D | V | L | S | W | S | F | A | S | K | 251 | P05087 |
| Phytohemagglutinin (*Phaseolus coccineus*) | 239 | G | N | V | E | T | N | D | I | L | S | W | S | F | A | S | K | 254 | Q8RW23 |
| Lectin precursor (*Phaseolus lunatus*) | 242 | G | N | V | E | T | H | D | V | L | S | W | S | F | A | S | K | 257 | Q9LED8 |
| Lectin precursor (*Vigna linearis var. latifolia*) | 244 | G | S | I | E | T | H | D | V | L | S | W | S | F | A | S | K | 259 | Q8L683 |
| Lectin precursor (*Dolichos biflorus*) | 239 | G | Y | T | E | T | H | D | V | L | S | W | S | F | A | S | K | 254 | P19588 |
| Lectin (*Acacia farnesiana*) | 196 | T | R | G | E | T | H | D | V | L | S | W | S | F | A | S | K | 211 | P84849 |
| Lectin (*Glycine max*) | 246 | G | S | V | E | T | H | D | V | | S | W | S | F | A | S | K | 261 | B4XQ48 |
| Lectin precursor (*Maackia amurensis*) | 247 | T | E | V | E | T | H | D | V | L | S | W | S | F | T | S | T | 262 | P93247 |

**Figure 4.7** Amino acid sequence from the tryptic fragments of the purified *C. amarissima* 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.

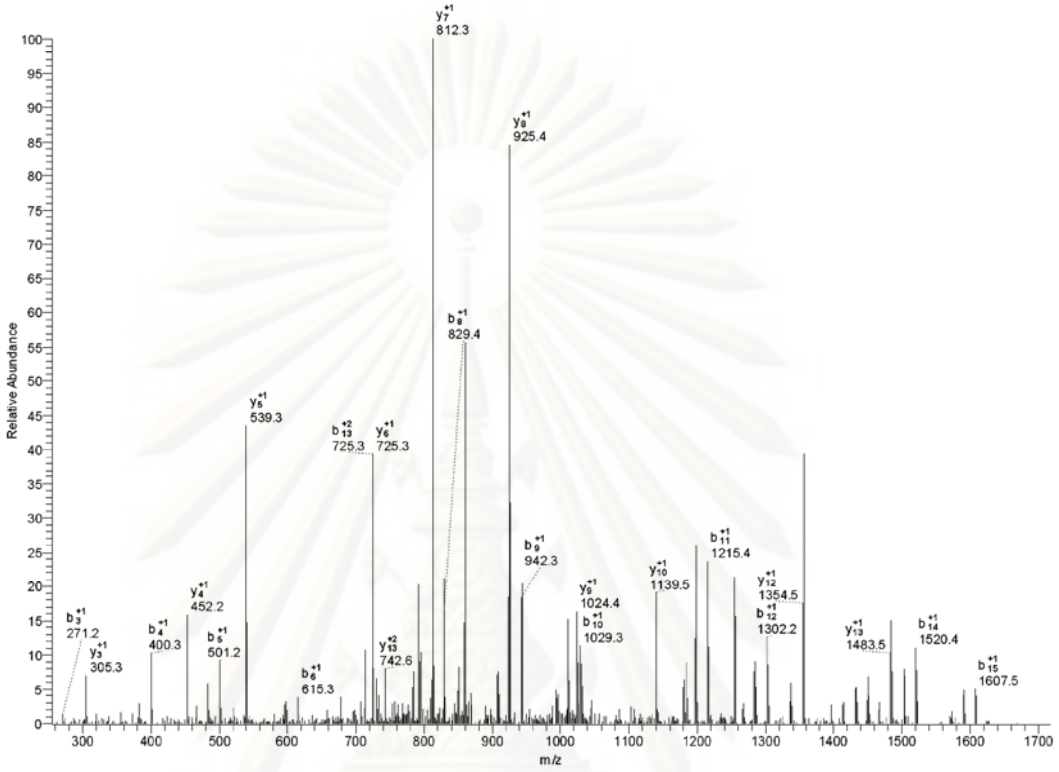


**Figure 4.8** LC/MS/MS spectra of the tryptic digest of the purified lectin used to derive the data in Figure 4.7 above.

### 4.8 Anti-proliferative activity

Lectins, and in particular plant lectins, have recently become of great interest since they have been reported as potential anticancer reagents that can seek out and stop multiplication of cancer cells. In this study, the *in vitro* antiproliferative and cytotoxic activity of the lectin isolated from the rhizomes of *C. amarissima* was evaluated against five human cancer cell lines, representing different organs and tissues, in tissue culture. This lectin was found to be active against the breast cancer cell line (BT474) at all the concentrations studied, and showed the highest antiproliferation activity with an $IC_{50}$ of ~21.2 μg, (Figure 4.9). These data are in agreement with the reported variation in antiproliferative potentials of a variety of

lectins with cancer cell lines, (Wang *et al*., 2000) and may be related to the sugar-binding activity of lectins where each specific carbohydrate chain, which is limited to the surface of tumor cells, acts as the receptor for one or more of the lectins (Mody *et al*., 1995). Several kinds of plant lectins have been reported to have antiproliferative effects upon tumor cell lines, such as *Cratylia mollis* lectin (Andrade *et al*., 2004) and *Viscum album* lectin. (Yoon *et al*., 2003)

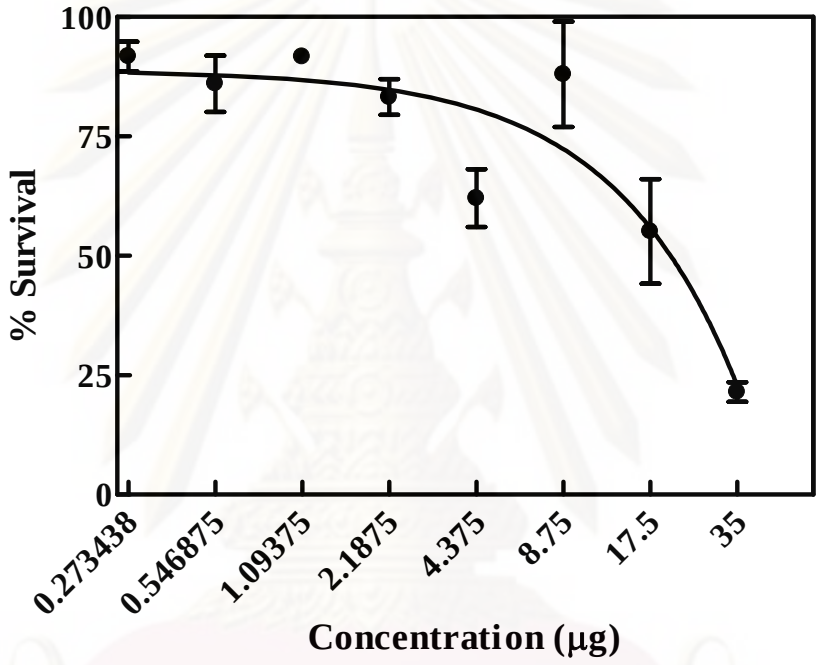


**Figure 4.9** Inhibitory activity of *C. amarissima* lectin towards the breast cancer cell line (BT474). Each data point represents the mean $\pm$1 S.D. of triplicate determinations.

**4.9 Anti-fungal activity**

The purified *C. amarissima* lectin showed *in vitro* antifungal activity against three plant pathogenic fungal species, *C. cassiicola E. turicicum* and *F. oxysporum*. It strongly inhibited the growth of *C. cassiicola* at 17.5 μg for *F. oxysporum* and *E. turicicum*, which were strongly inhibited at the higher concentration of 35 μg (Figure 4.10). Antifungal activity has been observed in other lectins where, for example

*Astragalus mongholicus* root lectin revealed antifungal activity against various species of phytopathogenic fungi (Yan *et al*., 2005). Similarly with lectin from *T. esculenta* seeds inhibited the growth of *F. oxysporum*, *C. lindemuthianum*, and *Saccharomyces cerevisiae*.(Freire *et al*., 2005) *In vitro* studies demonstrated that two novel chitin-binding lectins seeds of *Artocarpus integrifolia* inhibited the growth of *F. moniliforme* and *S. cerevisae* (Trindade *et al*., 2006). Many studies of plant lectins have assumed that they are implicated in host defense mechanism as antifungal proteins. However, to date only a small number of lectins have been reported to have actual antifungal activity such as lectin from the rhizomes of *Ophiopogon japonicus* showed antifungal activity against *Gibberella saubinetii* and *Rhizoctonia solani* (Tian *et al*., 2008). The purified *Phaseolus coccineus* Lectin (PCL) was devoid of antifungal activity against *C. albicans* and *P. italicum* (Chen *et al*., 2009).

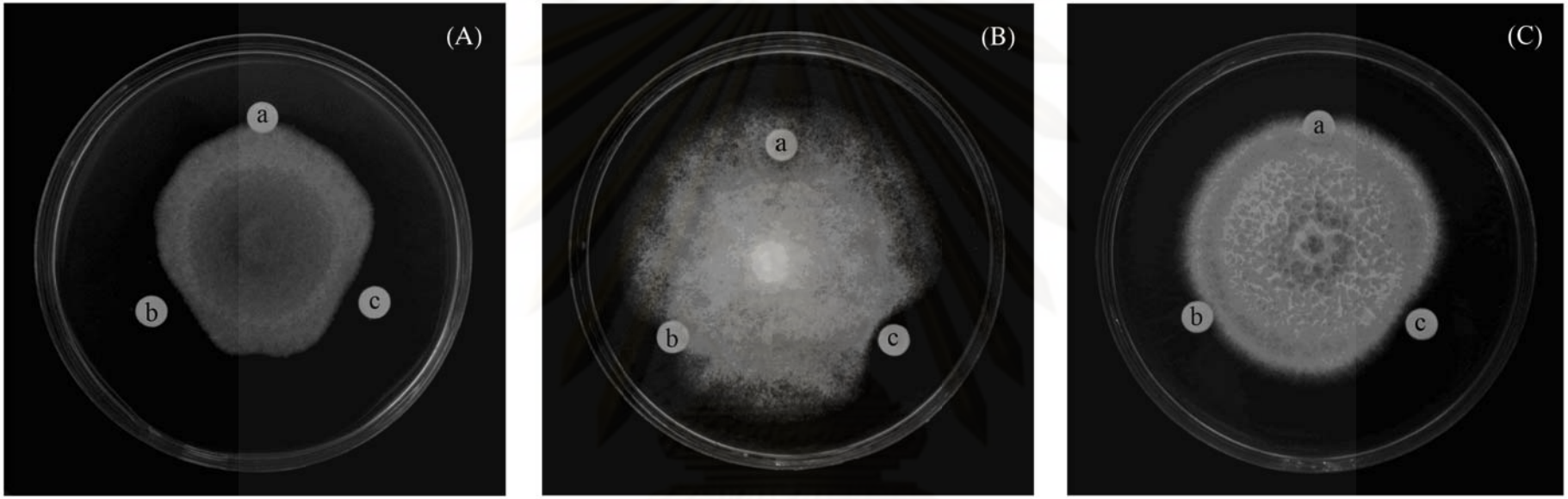


**Figure 4.10** Inhibitory effect of purified *C. amarissima* lectin on the *in vitro* growth on PDA plates (as an antifungal activity bioassay) of; (A) *C. cassiicola*, (B) *F. oxysporum* and (C) *E. turicicum*. For each plate, 0.625 cm diameter discs were seeded with 10 µl of TBS (a) alone as the negative control, or containing either (b) 17.5 µg/disc or (c) 35µg/disc purified *C. amarissima* lectin.

**4.10 Anti-microbial activity**

This research shown Lectin from *C. amarissima* inhibited 4 microbial growth consist of *B. subtilis, Candida albicans, E. coli*, and *S. aureus* at concentration ≥ 0.446, 0.446, 0.223, and 0.892 mg/ml respectively. But can not inhibite *P.auroginasa* growth because at the surface of *P. auroginosa* cell does not have polysaccharide ligands which can interact with *C. amarissima* lectin. Similar to Legume lectin from *Trinella foenumgraecum, Trifolium alexandrium, Bauhinia variegata,* and *Delonix regia* had a research that these lectins from sephadex G-150 can agglutinated both gram negative and gram positive bacteria (*Mycobacterium rhodochrous, B. cercur, B. megaterium, B. sphaericus, E. coli, Seratia marcescens, Corynebacterium xerosis,* and *S. aureus)* (Reda *et al*., 1992). Inaddition β-galactoside-binding lectin was extracted from the skin of amphibian, *Bufo arenarum*. It had an antimicrobial activity against Gram negative bacteria (*E. coli* K12 4100 and wild strains of *E. coli* and *Proteus morganii*) and Gram positive bacteria (*Enterococcus faecalis*) (Alicia *et al*., 2003)

**4.11 Alpha-glucosidase inhibition assay**

During the last few decades, there has been widespread interest in a-glucosidase (EC 3.2.1.20) because of its important role not only in carbohydrate digestion, but also in the processing of gluco-proteins and glyco-lipids. In addition, the alpha-glycosidase inhibitors have wide application for treatment of carbohydrate mediated diseases such as diabetes (Fujisawa *et al*., 1991), cancer (Humphries *et al*., 1986; Pili *et al*., 1995), (Shimizu *et al*., 1990), and certain forms of hyperlipoproteinemia and obesity. (Sou *et al*., 2001). The alpha-glucosidase enzyme is required for the breakdown of carbohydrates to absorbable mono-saccharides at the intestine. The alpha-glucosidase inhibitor is usually used to prevent or medically treat type II diabetes (Non-insulin-dependent diabetes mellitus (NIDDM). These inhibitors combine with intestine alpha-glucosidase and block the uptake of postprandial blood glucose. (Holman, 1998)

Half Inhibition Concentration ($IC_{50}$) of *C. amarissima* lectin is about 0.073 mg/ml of protein this study used acarbose solution as a control. The $IC_{50}$ of acarbose is 0.022 mg/ml of glucose. In addition the lectin from kidney beans (*Phaseolus vulgaris*) could inhibit the alpha-glucosidase enzyme too. This lectin from canned beans had inhibition for alpha-glucosidase is 70.6%. The raw kidney beans could

inhibite the alpha-glucosidase enzyme about 77.1%.(Shi *et al*., 2007). Moreover, protein extracts of *Sesbania grandiflora* flowers had a report that were alpha-glucosidase inhibitor proteins. The crude extract of proteins from 60% and 90% saturation precipitation had the inhibition for alpha-glucosidase were 49.55% and 82.07%, respectively. In the recent year, the alpha-glucosidase inhibitor from lectin was rarely report (Boonmee *et al*., 2007).

# CHAPTER V

# CONCLUSION

In this study, a lectin from the rhizomes of *C. amarissima* was purified by affinity and gel filtration chromatography to apparent homogeneity. The molecular mass of the purified lectin was 32.4 kDa, as estimated by SDS-PAGE, and although not non-specific, it showed no single target or distinctive specificity in its ability to hemagglutinate erythrocytes from either different human blood groups (A, B, AB and O), or from different animals where it agglutinated erythrocytes from rabbits, humans and, weakly, rats, but not mice, guinea pigs, geese and sheep. The lectin was thermostable up to 40 $^{o}$C and showed an optimum activity within the pH range of 8.0-11.0. Divalent cations appear to be essential for the hemagglutination activity of this lectin. The deduced internal amino acid sequence of the lectin showed similarity (homology) to the sequences of the leucoagglutinating phytohemagglutinin precursor lectin family. The lectin was able to inhibit the *in vitro* growth of the plant pathogenic fungi, *E. turcicum*, *F. oxysporum* and *C. cassicola*, within the range of 17.5-35 μg/ml and four microbial, *B. subtilis*, *C. albican*, *E. coli*, and *S. aureus* at concentration ≥ 0.446, 0.446, 0.223, 0.892 mg/ml, respectively. Furthermore, this *C. amarissima* rhizome derived lectin showed *in vitro* cytotoxicity against the BT474 human breast cancer cell line with an $IC_{50}$ of ~21.2 μg/ml. Additionaly, $IC_{50}$ for an alpha-glucosidase activity is 0.073 mg/ml protein or 0.024 mg/ml of glucose.

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# APPENDICES

# APPENDIX A

## 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 | 1,000 | ml |

Boil 200 g of peels, dried potato for 1 hr in 1000 ml. of distilled water. Filter, and make up the filtrate to one liter. Add the glucose and agar and dissolve by streaming.

2. NA (Nutrient Agar)

| | | |
|---|---|---|
| Peptone | 5.0 | g |
| Beef extract | 3.0 | g |
| Agar | 20.0 | g |
| Distilled water | 1,000 | ml |

3. NB (Nutrient Broth)

| | | |
|---|---|---|
| Peptone | 5.0 | g |
| Beef extract | 3.0 | g |
| Distilled water | 1,000 | ml |

4. YMA (Yeast Malt Agar)

| | | |
|---|---|---|
| Yeast extract | 3.0 | g |
| Malt extract | 3.0 | g |
| Glucose | 10.0 | g |
| Peptone | 5.0 | g |
| Agar | 20 | g |
| Distilled water | 1,000 | ml |

5. YMB (Yeast Malt Broth)

| | | |
|---|---|---|
| Yeast extract | 3.0 | g |
| Malt extract | 3.0 | g |
| Glucose | 10.0 | g |

| | | |
|---|---|---|
| Peptone | 5.0 | g |
| Distilled water | 1,000 | ml |

# APPENDIX B

**Preparation for denaturing polyacrylamide gel electrophoresis**

**1. Stock solutions**

**2 M Tris-HCl (pH 8.8)**

Tris (hydroxymethyl)-aminomethane 24.2 g

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

**1 M Tris-HCl (pH 6.8)**

Tris (hydroxymethyl)-aminomethane 12.1 g

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

**10% SDS (w/v)**

Sodium dodecyl sulfate (SDS) 10 g

**50% Glycerol (w/v)**

100% Glycerol 50 ml

Added 50 ml of distilled water

**1% Bromophenol blue (w/v)**

Bromophenol blue 100 mg

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

Filtration will remove aggregated dye.

**2. Working solution**

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

Acrylamide 29.2 g

| | |
|---|---|
| N,N,-methylene-bis-acrylamide | 0.8 g |
| Adjust volume to 100 ml with distilled water | |

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

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

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

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

**10% Ammonium persulfate**

| | |
|---|---|
| Ammonium persulfate | 0.5 g |
| Distilled water | 5 ml |

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

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

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

| | |
|---|---|
| 1 M Tris-HCl (pH 6.8) | 0.6 ml |
| Glycerol | 5 ml |
| 10% SDS | 2 ml |
| 1% Bromophenol blue | 1 ml |
| 2-mercaptoethanol | 0.5 ml |

| | | |
|---|---|---|
| Distilled water | 0.9 | ml |

**3. SDS-PAGE**

**15% Separating gel**

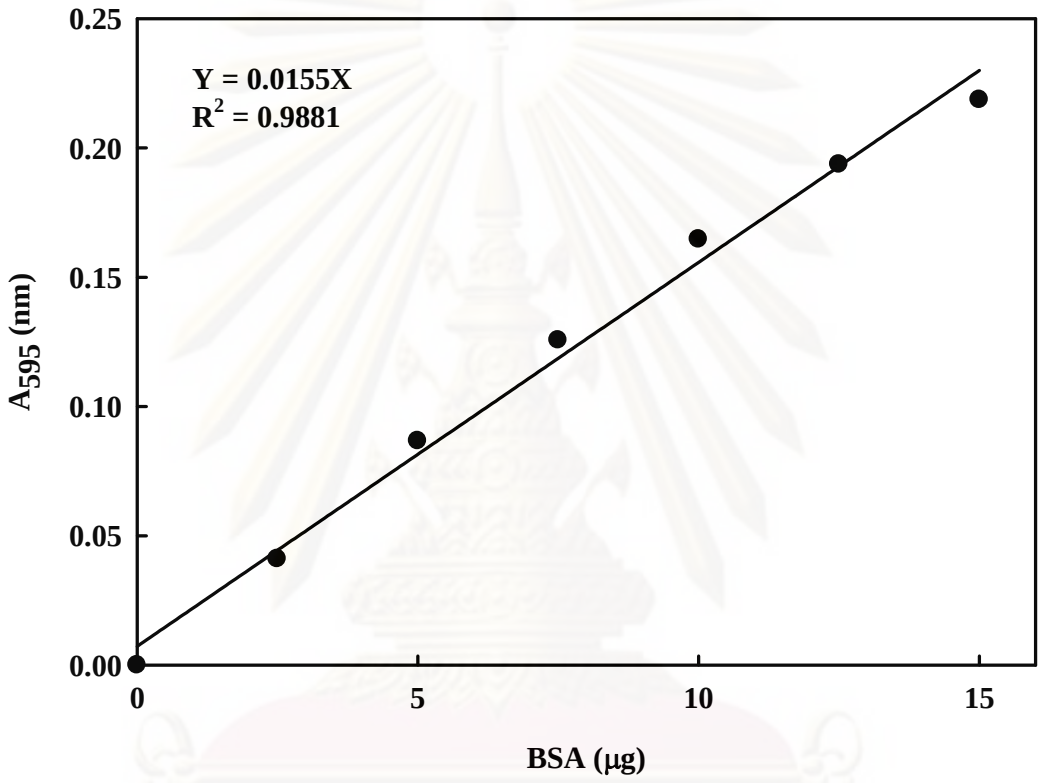
| | | |
|---|---|---|
| Solution A | 10.0 | ml |
| Solution B | 5.0 | ml |
| Distilled water | 5.0 | ml |
| 10% Ammonium persulfate | 100 | µl |
| TEMED | 10 | µl |

**5.0% Stacking gel**

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

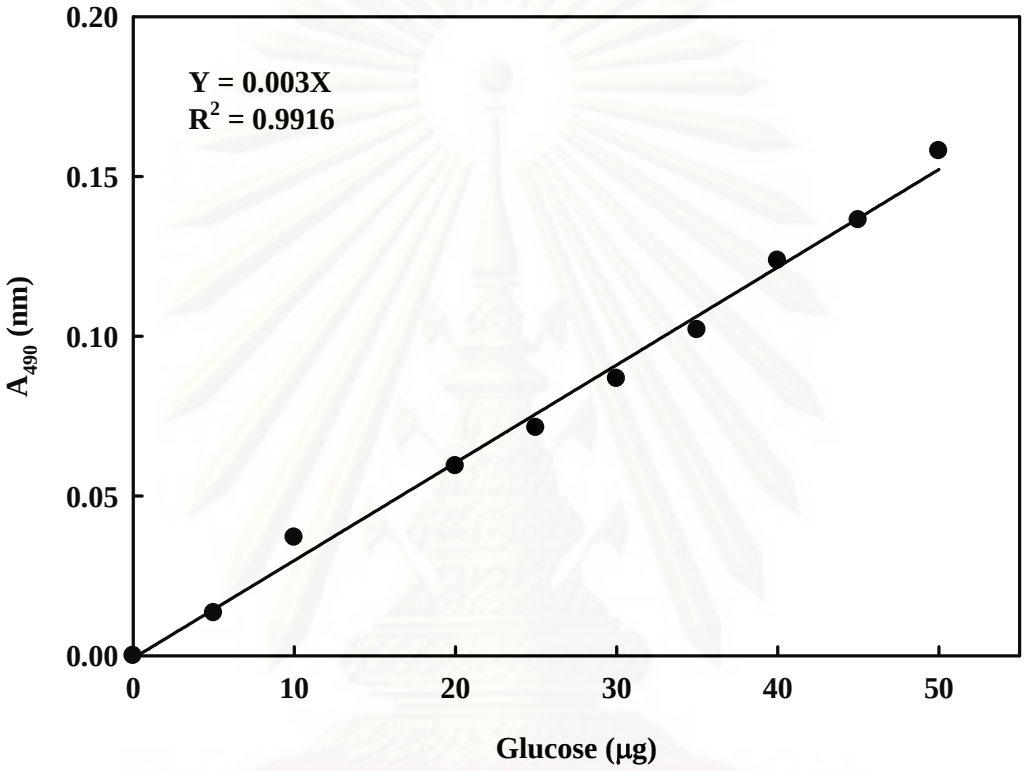
# APPENDIX C

**Calibration curve for protein determination by Bradford method**



# APPENDIX D

**Calibration curve for carbohydrate content by Dubois method**



# APPENDIX E

**Amino acid abbreviations**

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

# BIOGRAPHY

Miss Norhameemee Kheeree was born on February 27, 1985 in Narathiwat, Thailand. She graduated with Bachelor Degree of Science from Department of Biotechnology, Faculty of Science, Thammasat University in 2006. She was admitted to the Master degree of Science in Biotechnology, Faculty of Science, Chulalongkorn University in 2007.

**Academic presentation;**

1) Kheeree, N., Sangvanich, P., Puthong, S., and Karnchanatat, A. A lectin from the rhizomes of *Curcuma amarissama* Roscoe. and its role as anticancer 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.

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