การทำให้บริสุทธิ์และลักษณะสมบัติของเลคตินจากเมล็ดลูกเนียง *Archidendron jiringa* Nielsen.

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PURIFICATION AND CHARACTERIZATION OF LECTIN FROM *Archidendron jiringa* Nielsen. SEEDS

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ในงานวิจัยนี้ได้ทำการศึกษาลักษณะสมบัติของเลคตินจากเมล็ดของลูกเนียง *Archidendron jiringa* Nielsen. โดยการนำเมล็ดลูกเนียงมากำจัดไขมันด้วยแอซิโตนแล้วสกัดด้วยสารละลายทริสบัฟเฟอร์พีเอช 7.2 จากนั้นนำโปรตีนมาทำให้บริสุทธิ์โดยการตกตะกอนด้วยเกลือแอมโมเนียมซัลเฟต 90 เปอร์เซนต์ และแยกโดยเทคนิคโครมาโทกราฟีแบบสัมพรรคภาพด้วยคอลัมน์คอนเอ-เซฟาโรส เมื่อใช้เทคนิคพอลิอะคริลาไมด์เจลอิเล็กโตรฟอเรซิสแบบเสียสภาพ เลคตินที่ได้มีน้ำหนักโมเลกุลประมาณ 35.7 กิโลดาลตัน เลคตินที่ได้มีสมบัติในการทำให้เม็ดเลือดแดงในกระต่าย หนูแรท หนูเม้าส์ หนูตะเภา ห่าน แกะ และคนในหมู่เลือด ABO มีการเกาะกลุ่มกัน เลคตินมีกิจกรรมการเกาะกลุ่มกันของเม็ดเลือดแดงสูงที่สุดที่ค่าความเป็นกรด-ด่างเท่ากับ 8 และที่อุณหภูมิต่ำกว่า 45 องศาเซลเซียสเป็นเวลา 30 นาที กิจกรรมการเกาะกลุ่มกันของเม็ดเลือดแดงลดลง 50 เปอร์เซนต์ที่อุณหภูมิ 40 องศาเซลเซียสเป็นเวลา 120 นาที และลดลงอย่างรวดเร็วที่อุณหภูมิ 70 องศาเซลเซียส กิจกรรมการเกาะกลุ่มกันของเม็ดเลือดแดงถูกกระตุ้นได้ด้วยไอออนของแคลเซียม แมกนีเซียม และแมงกานีส เมื่อวิเคราะห์กรดอะมิโนภายในของเลคตินพบว่าอยู่ในแมนโนส-กลูโคสสเปซิฟิกเลคตินแฟมิลี เลคตินที่ได้สามารถยับยั้งการเจริญเติบโตของเชื้อราโรคพืช *Fusarium oxysporum Exserohilum turcicum* และ *Colletotrichum cassiicola* ได้ที่ความเข้มข้นมากกว่า 5.66 ไมโครกรัม เลคตินปริมาณ 4.97 ไมโครกรัมสามารถยับยั้งแอลฟากลูโคซิเดสได้ 52.29 เปอร์เซนต์ และเลคตินไม่มีความเป็นพิษต่อเซลล์มะเร็ง

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SARINYA CHARUNGCHITRAK : PURIFICATION AND CHARACTERIZATION OF LECTIN FROM *Archidendron jiringa* Nielsen. SEEDS. ADVISOR : ASSOC. PROF. AMORN PETSOM, Ph.D., CO-ADVISOR : APHICHART KARNCHANATAT, Ph.D., 67 pp.

In this research, a plant lectin from seeds of *Archidendron jiringa* Nielsen. was extracted with Tris-HCl pH 7.2 buffer containing 0.15 M NaCl after the seed was defatted with acetone. The protein was precipitated with 90% ammonium sulfate and purified using affinity chromatography on ConA Sepharose. The molecular mass of purified lectin was 35.7 kDa as estimated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The purified lectin showed no specificity in its ability to hemagglutinate human (A, B, AB and O) erythrocytes and indiscriminately agglutinate rabbit, rat, mouse, guinea pig, goose and sheep. Hemagglutination activity of lectin was markedly affected at pH 8. It was heat stable below 45°C for 30 min. The activity was decreased to 50% when heated at 40°C for 120 min and rapidly fully inactivated at 70°C. It was found that *A. jiringa* lectin required divalent metal cations (Ca^{2+}, Mg^{2+}, and Mn^{2+}) for hemagglutination activity. The purified lectin had an internal amino acid sequences composition which was similar to that of mannose-glucose specific lectin family. The purified *A. jiringa* lectin inhibited growth of *Fusarium oxysporum*, *Exserohilum turicicum* and *Colectrotrichum cassiicola* at the concentration of > 5.66 μg. The 4.97 μg of purified lectin was led to the 52.29% of α-glucosidase inhibitory activity. *A. jiringa* lectin showed no cytotoxicity for five cell lines.

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CONTENTS

	Page
ABSTRACT (THAI) ……..……..…	iv
ABSTRACT (ENGLISH)……..…….....	v
ACKNOWLEDGEMENTS……...……...…….	vi
CONTENTS……...……...…….	vii
LIST OF TABLES……..……...…….	x
LIST OF FIGURES……...…….	xi
LIST OF ABBREVIATIONS……...…….	xiii
CHAPTER	
I INTRODUCTION……..……...…….	1
II THEORETICAL……...…….	3
2.1 Lectins……...……...…….	3
2.1.1 Definition of plant lectins……...……...…….	3
2.1.2 Lectin sources…….……...…….	3
2.1.3 Biological activities of lectins……...…….	4
2.1.3.1 Cell agglutination…….……...…….	4
2.1.3.2 Carbohydrate specificity……..…...…….	5
2.1.3.3 Antibacterial activity……...…….	6
2.1.3.4 Antifungal activity……...……...…….	7
2.1.4 Potential applications of lectins…….…...…….	7
2.2 Legume lectins……...……...…….	8
2.2.1 Occurrence……...….....	8
2.2.2 Carbohydrate-Binding Specificity…….…...…….	9
2.2.3 Physiological Role…….……...…….	10

CHAPTER Page

2.3 Separation Techniques……...……...……. 11
2.3.1 Affinity chromatography……...……. 11
2.3.2 Sodium dodecyl sulfate polyacrylamide gel electrophoresis……. 15
2.4 Protein Identification Techniques…….……. 17
2.4.1 Mass spectrometry……..……... 17
2.4.1.1 Electrospray Ionization……...……. 17
2.4.1.2 Matrix assisted laser desorption ionization……..…… 19
2.5 Protein identification by database searching……....……. 20
2.6 Literature review……...……. 21

III EXPERIMENTAL……...…...……. 23

3.1 Biological materials……..……...…….…. 23
3.2 Chemicals and reagents……..……...……. 23
3.3 Apparatus and Instruments……..……..……. 24
3.4 Procedures……....……. 24
3.4.1 Extraction of *A. jiringa* seeds lectin……..……. 24
3.4.2 Purification of *A. jiringa* seeds lectin……....……. 24
3.4.3 Protein determination…….…...……. 25
3.4.4 Hemagglutination assays……...…. 25
3.4.5 SDS polyacrylamide gel electrophoresis……....……. 25
3.4.6 Effect of temperature on lectin hemagglutinating activity and thermostability……...……...……. 26
3.4.7 pH-dependence of agglutination activity……...…...……. 26
3.4.8 Effect of metal ions……...……. 26
3.4.9 Internal amino acid sequence of lectin by LC–MS/MS……. 26
3.4.10 Assay of antifungal activity……... 27
3.4.11 Assay for alpha glucosidase inhibitor……...……. 27
3.4.12 Cytotoxicity test against cancer cell lines…….……..….. 28

CHAPTER Page

IV RESULTS AND DISCUSSION……...….... 29

4.1 Purification of lectin from seeds of *A. jiringa* …….. 29
4.2 SDS-PAGE analysis……... 31
4.3 Assay for Hemagglutinating activity……. 32
4.4 Effect of temperature on lectin activity and stability……. 34
4.5 Effect of pH on lectin activity……..... 36
4.6 Effect of metal ions……..….. 37
4.7 Protein identification by LC-MS/MS……. 37
4.8 Assay of antifungal activity……..... 40
4.9 Assay for alpha glucosidase inhibitor…… 42
4.10 Cytotoxicity test against cancer cell lines……...….... 42

V CONCLUSION……...…….. 43
REFERENCES……...……..... 45
APPENDICES……...……..... 55
VITA……...……... 67

LIST OF TABLES

Table		Page
4.1	Purification table of the lectin from seeds of *A. jiringa*……....	30
4.2	Hemagglutinating activity of *A. jiringa* against human and animal erythrocytes……...…....	33
4.3	Effect of some cations on purified lectin from *A. jiringa* seeds….....	37

LIST OF FIGURES

Figure		Page
1.1	*Archidendron jiringa* seed……..……..…..…	2
2.1	Schematic representation of cell agglutination by a lectin and of hapten inhibition of the agglutination. ……..…..…….....…	5
2.2	Structures of monosaccharides on cell surface of microorganisms, which are specific for lectin interaction. ……..……	6
2.3	A) Affinity medium is equilibrated in binding buffer. B) Sample is applied under conditions that favor specific binding of the target molecule(s) to a complementary binding substance (the ligand). Target substances bind specifically, but reversibly, to the ligand and unbound material washes through the column. C) Target protein is recovered by changing conditions to favor elution of the bound molecules. Elution is performed specifically, using a competitive ligand, or non-specifically, by changing the pH, ionic strength or polarity. Target protein is collected in a purified, concentrated form. D) Affinity medium is re-equilibrated with binding buffer……	13
2.4	Affinity chromatogram……..	14
2.5	SDS-PAGE……	16
2.6	A full diagram of a mass spectrometer……..	17
2.7	Electrospray ionization source ……..…….……..	18
2.8	The electrospray ionization process…….……...…..	18
2.9	Matrix assisted laser desorption ionization (MALDI)……...	20
4.1	Affinity chromatogram of lectin on ConA Sepharose column (1.6x20 cm) equilibrated with 20 mM Tris-HCl pH 7.2 containing 0.15 M NaCl. Lectin was eluted with 20 mM Tris-HCl pH 7.2 containing 0.15 M NaCl, 0.2 M Methyl-α-D-glucopyranoside at a flow rate of 1.5 ml/min……...……...….	31
4.2	SDS-PAGE analysis of lectin. Lane 1, molecular weight standards; Lane 2 purified lectin from seeds of *A. jiringa*…….	32

Figure Page

4.3 A) Effect of temperature on the agglutinating activity of lectin towards rabbit erythrocyte suspension. The 50 mM Tris-HCl, pH 7.2 buffer system was used. B) Thermostability of purified lectin towards rabbit erythrocyte suspension. The 20 mM Tris-HCl, pH 7.2 buffer system was used: (○) 40°C; (●) 50°C; (■) 60°C; and (▲) 70°C. Each point on the line represents the average of three replicates. Full activity (100%) corresponded to a titer of 2^4…….…. 35

4.4 Effect of pH on the agglutinating activity of lectin towards rabbit erythrocyte suspension. The 20 mM Tris-HCl, pH 7.2 buffer system was used. Each point on the line represents the average of three replicates. Full activity (100%) corresponded to a titer of 2^7…..……... 36

4.5 Amino acid sequences from the fragment obtained by tryptic digestion of the purified lectin from *A. jiringa* seeds. Comparisons are made with other lectin classified as mannose-glucose specific lectin family. Shaded regions represent regions of identity…….....…… 38

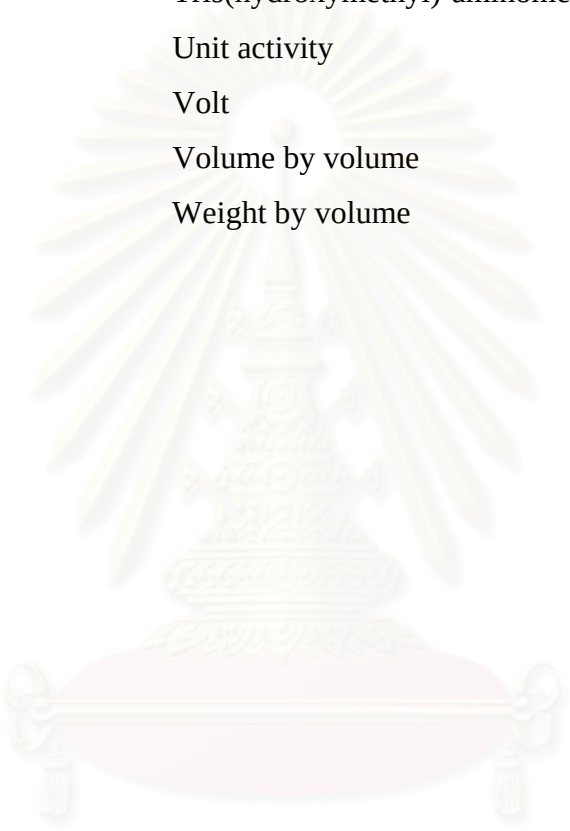
4.6 MS-MS spectra of purified lectin from *A. jiringa* seeds…….…….. 39

4.7 Inhibitory effect of *A. jiringa* lectin on antifungal protein toward A) *C. cassiicola*, B) *F. oxysporum*, and C) *E. turicicum*. The negative control is 10 µl of (a) 20 mM Tris-HCl buffer pH 7.2, (b) 5.66 µg *A. jiringa* lectin, and (c) 11.31 µg *A. jiringa* lectin…… 41

LIST OF ABBREVIATIONS

1-D electrophoresis	One-dimensional electrophoresis
µg	Microgram
µl	Microlitre
APS	Ammonium persulfate
A	Absorbance
Bis	*N*,*N*'-methylenebisacrylamide
BSA	Bovine serum albumin
°C	degree Celsius
cm	Centimeter
Da	Dalton
DMSO	Dimethyl sulfoxide
EDTA	Ethylenediaminetetraacetic acid
ESI	Electrospray ionization
g	Gram
hr	Hour
kDa	Kilodaton
l	Litre
M	Molar
MALDI	Matrix Assisted Laser Desorption Ionization
mA	Milliampere
mg	Milligram
min	Minute
ml	Millilitre
MS	Mass spectrometry
MS/MS	Tandem Mass spectrometry
m/z	Mass per charge
mM	Millimolar
MW	Molecular weight
N	Normal
nm	Nanometer

PAGE	Polyacrylamide-gel electrophoresis
ppm	Parts per million
rpm	Revolutions per minute
SDS	Sodium-dodecyl sulfate
TEMED	*N*,*N*,*N*',*N*'-tetramethylethylenediamine
Tris	Tris(hydroxymethyl)-aminomethane
U	Unit activity
V	Volt
V/V	Volume by volume
W/V	Weight by volume



CHAPTER I

INTRODUCTION

Lectins are proteins or glycoproteins of ubiquitous distribute in nature, which have at least one carbohydrate or derivative binding site without catalytic function or immunological characteristics. It has the unique ability to recognize and bind reversibly to specific carbohydrate ligands without any chemical modification that distinguishes lectins from other carbohydrate binding protein and enzymes and make them invaluable tools in biomedical and the glycoconjugate research [1].

Plant is the first source of lectin discovery and most frequently used in lectin studies due to the ease of extraction and the yield obtained. Lectins can be found widespread throughout the plant kingdom in different families as well as different tissue in the same plants which contain lectins different molecular properties and variety of carbohydrate specificities. It indicated that plant lectins have important roles according to their abundance. Lectins have been adapted for several functions during evolution [2].

The role of lectin in defense mechanism of plants may have evolved from the ability of lectins to agglutinate and immobilized microorganism. A review summarized the supporting evidences for this proposed role into two main observed actions; a) a presence of lectin at the potential sites of invasion by infections agent [3], b) the binding of lectins to various fungi and their ability to inhibit fungal growth and germination [4].

A number of studies with respect to plant defense role of lectins have been reported. For example, during the imbibitions of dry soybean seeds, lectin is released into water. The presence of lectin in vicinity of germinating seeds hint possible interaction of lectin with potential pathogen. The developmental pattern of initial accumulation and final disappearance of lectin can be observed during the seed dormacy, germination, and maturation. This evidence may implicate the role of lectin in a defense mechanism necessary for plant in the initial stage of growth [5].

Archidendron jiringa Nielsen. is a plant in Mimosaceae family, of the Archidendron genera, that found in the southern part of Thailand and it is an economically important species for having multiplicity uses. The seeds (cotyledons) are normally eaten raw at mealtimes and believe to help purify the blood [6].

Figure 1.1 *Archidendron jiringa* seed [7].

Objectives

The objective of this research was to isolate and characterized lectin from *A. jiringa* seeds and investigated its biological activity.

Scope of work

In initial work, seeds of *A. jiringa* was extracted and separated by chromatography and electrophoresis technique. Afterwards, purified lectin was identified by mass spectrometer and database searching.

CHAPTER II

THEORETICAL

2.1 Lectins

2.1.1 Definition of plant lectins

A lectin is a sugar-binding protein of non-immune origin that agglutinates cells or precipitates glycoconjugates. A lectin molecule contains at least two sugar-binding sites; sugar-binding proteins with a single site will not agglutinate or precipitate structures that contain sugar residues, so are not classified as lectins. The specificity of a lectin is usually defined by the monosaccharides or oligosaccharides that are best at inhibiting the agglutination or precipitation the lectin causes. Lectins occur in many types of organism; they may be soluble or membrane-bound; they may be glycoproteins. Sugar-specific enzymes, transport proteins and toxins may qualify as lectins, if they have multiple sugarbinding sites [8].

2.1.2 Lectin sources

Lectins are widely distributed in nature, and occur in diverse organisms ranging from plant, animal, fungi, bacteria, and virus [9]. Legumes and monocots are major sources of plant lectins that have been widely studied [10]. Plant lectins can be classified into four major families of structurally and evolutionary related proteins: legume lectins, type 2 ribosome inactivating proteins, chitin-binding lectins, and monocot mannose-binding lectins [11, 12, 13]. Three other small lectin families (Cucurbitaceae phloem lectins, amaranthins, and jacalin-related lectins) have also been characterized [11].

Legume lectins represent the largest and most thoroughly studied family of plant lectins. They have been isolated from seeds, stem, and bark of legumes [14]. The best known legume lectins are phytohemagglutinin (PHA) from red kidney bean, soybean (SBA), jackbean (concanavalin A), peanut lectin (PNA), and pea (PSL) [15].

2.1.3 Biological activities of lectins

From their carbohydrate specificity, lectins can agglutinate cells, for example, erythrocytes resulting in hemagglutination activities [16]. Lectins have been implicated in various biological activities as a result of their recognition of carbohydrates [15].

2.1.3.1 Cell agglutination

Each lectin molecule, which generally contains two or more carbohydrate binding sites, can interact with cells by combining to sugars on their surface, thus, cross-linking the cells and resulting in the phenomena of cell agglutination and their subsequent precipitation (Figure 2.1) [17]. So the red blood cell agglutination or hemagglutination of lectins is the major attribute of these proteins, and used routinely for their detection and characterization. Lectins also form cross-links between polysaccharides or glycoprotein molecules in solution, and induce their precipitation reaction [16].

Agglutination is a complex process that is affected by several internal factors such as molecular properties of lectin (e.g. molecular size, number of carbohydrate binding sites, binding affinity, cell surface properties, number and accessibility of lectin to binding sites, metabolic state of cell) [18]. Additionally, conditions occur during agglutination process, in particular, temperature, type of cell and lectin used, and cell concentration, may also be considered as external parameter effects for the agglutination. The agglutination and precipitation activities of lectin are similar to antibodies which can be inhibited by low molecular weight compound called haptens. In this case sugars perform as hapten for inhibition assay for indication of carbohydrate structure that the lectin is specific for, and are present on the cell surface [17] (Figure 2.1).

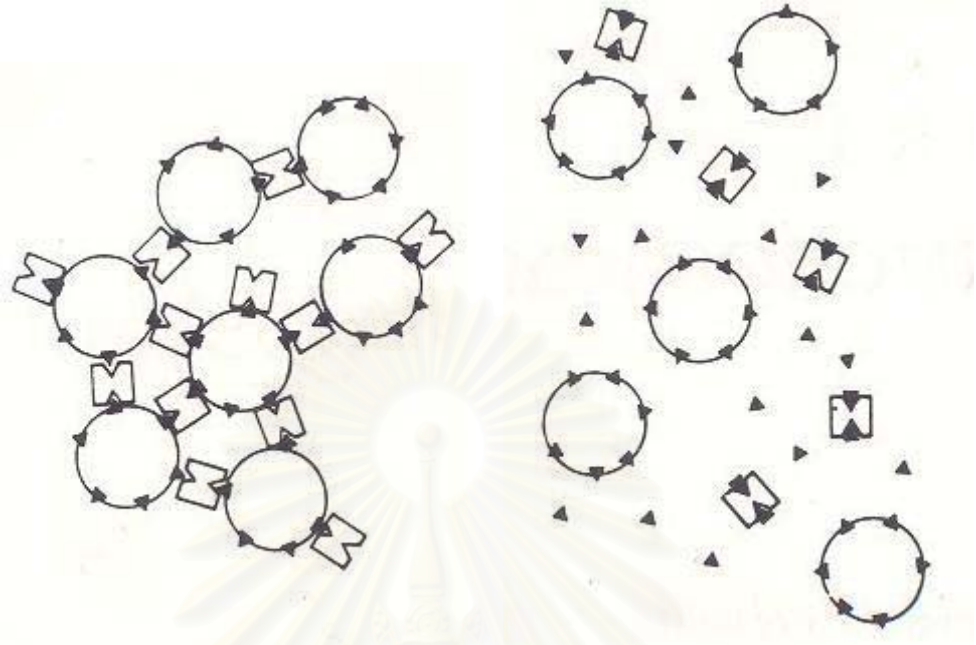


Figure 2.1 Schematic representation of cell agglutination by a lectin and of hapten inhibition of the agglutination. Source: Sharon and Lis [17]

2.1.3.2 Carbohydrate specificity

On the basis of their specificity, lectins are classified into 5 specificity groups according to the monosaccharide that they exhibit the highest affinity with; galactose/N-acetylgalactosamine, N-acetylglucosamine, N-acetylneuraminic acid, fucose, and mannose. Relevant for the biological activities of the lectins is the fact that of the numerous monosaccharides found in nature, only those listed above are typical constituents of surfaces of eukaryotic cells (Figure 2.2) [15]. The binding affinities of lectins are much lower than enzyme and it is also dynamic and reversible. The binding constant of lectin-monosugars interactions is relatively low (in the mM range) and lower than lectin oligosaccharide interactions [18]. This is due to the oligosaccharides interacting stronger with various secondary binding sites. Throughout nature, galactose and N-acetylgalactosamine specific lectins appear to be the most abundance, followed by mannose specific lectin [15]. In recent years, numerous oligosaccharides and glycoproteins of known structures have been widely employed for defining the specificity of lectins that interact poorly or not at all with any monosaccharide.

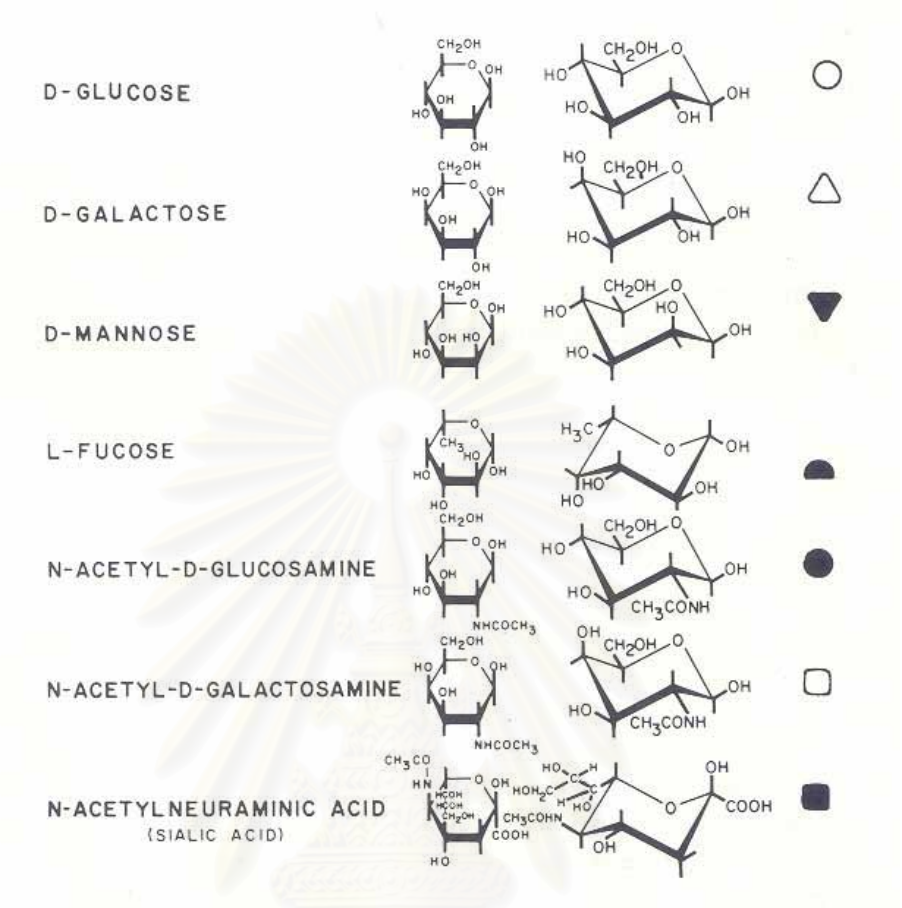


Figure 2.2 Structures of monosaccharides on cell surface of microorganisms, which are specific for lectin interaction. Source: Sharon and Lis [17]

2.1.3.3 Antibacterial activity

Owing to the physiological functions of plant lectins in nature, antibacterial activity of lectins has been intensively discussed. However, only a few researches on antibacterial lectins have been published. Antibacterial activity of cell wall lectins from potato has been reported, and it was found that the lectins can immobilized only avirulent strains of *Pseudomonas solanacearum*. However, they cannot recognize virulent strains at all [19]. Another study has been conducted with the apple (*Datura stramonium*) lectins [20]. The lectins mediated blocking of bacterial motility *in vitro*.

2.1.3.4 Antifungal activity

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 antifungal activity. The antifungal properties of plant lectins have been found from potato tuber [19], *Amaranthus caudatus* seed [21], stinging nettle rhizomes [22], wheat germ, and recently from *Phaseolus vulgaris* seed [23]. Some of them are chitin binding lectins. The most potent antifungal lectin belongs to class I chitinase [24]. Eventually, the antifungal activity assay was carried out in the higher fungi; *Agrocybe cylindracea*, *Agaricus bisporus* [25] and *Lyophyllum shimeiji* lectins [26]. Manifest antifungal activities have not been demonstrated. Thus the discovery of antifungal lectins from plants and also higher fungi are of interest.

2.1.4 Potential applications of lectins

Lectins are applied predominantly according to their carbohydrate recognition which is based on precipitation and agglutination reaction [15]. They have been widely applied in scientific research particularly in clinical, biochemical, and biotechnology studies [27]. Several of them have been commercially utilized [28].

In 1963, Aubb, Burger and others discovered that a plant lectin, wheat germ agglutinin, has selectively agglutinating property to murine tumor cells. It has been revealed that neoplastic cells are differing from normal cells at the glycoconjugates on the cell surface. For example, diagnosis of cancer by mitogenic index, *in vitro* selection of antitumor cell cytotoxicity, *in vivo* reduction of cancer, efficient vaccines and immunomodulation against bacterial infection [27, 29]. Beside that the endogenous lectins from animal have been used as drug or drug targets. These lectins include CD33, which were targeted for *in vivo* leukemic blast reduction; CD44, for both differentiation therapy in acute myeloid leukaemia and as a selective drug delivery system.

2.2 Legume lectins

The term ‘legume lectin’ refers to a particular type of plant lectin that is found exclusively in the Leguminoseae. It should be emphasized that not all lectins found in legume species belong to the legume lectins. For example, the class I chitinase from bean (*Phaseolus vulgaris*) and the seed lectins from *Abrus precatorius* belong to the chitin-binding lectins and type 2 RIP, respectively. Legume lectins are not only the most famous lectin family but also played a determining role in the development of lectinology as a scientific discipline. In addition, several legume lectins have become indispensable tools in biological and biomedical research and are widely used as bioactive proteins in research and medicine [30].

2.2.1 Occurrence

Legume lectins are a large family of closely related proteins that heretofore have been found exclusively in representatives of the Leguminoseae (Fabaceae) [31]. At present about 100 legume lectins have been isolated from over 70 different species belonging to various taxonomic groups. Most legume lectins have been isolated from mature seeds. Typical legume lectins usually account for 1 to 10% of the total soluble seed protein. In some species higher lectin concentrations (up to 50%) have been observed, whereas in others the lectin represents less than 0.1% of the total seed protein. Several legumes contain two or more different seed lectins. Taking into consideration that some seed lectins are very rare proteins, the occurrence of multiple lectins in legume seeds is probably the rule rather than the exception. Legume seed lectins are predominantly located in the storage parenchyma cells of the cotyledons and to a lesser extent in the primary axes. Localization studies further indicated that legume seed lectins are typically sequestered in storage protein vacuoles (also known as protein bodies) [32].

Legume lectins have also been found in different vegetative tissues. Although the lectin concentration is usually (very) low in leaves, stems, roots and root nodules, lectins are the most abundant proteins (representing 20 to 50% of the total soluble protein) in the bark of several legume trees (e.g., *Robinia pseudoacacia*,

Maackia amurensis, and *Sophora japonica*). However, it should be noted that the lectin concentration in these tissues is developmentally regulated [33, 34]. Vegetative tissues can also contain two or more different lectins. For example, the bark of *Robinia pseudoacacia* [35] and *Sophora japonica* [36] contains three different lectins that are encoded by different genes. It is noteworthy that in all cases studied thus far (i.e., *Robinia pseudoacacia*, *Maackia amurensis*, and *Sophora japonica*), the bark and seed lectins are encoded by a different set of genes [37]. Localization studies have demonstrated that the abundant bark lectins are like their seed counterparts located predominantly in the storage parenchyma cells and, in addition, are also sequestered in subcellular organelles comparable to the seed storage protein vacuoles.

2.2.2 Carbohydrate-Binding Specificity

Legume lectins strongly differ from each other with respect to their carbohydrate-binding specificity. A brief overview of the sugar-binding specificities of the currently known plant lectins indicates that the legume lectins cover a much broader range of specificities than any other lectin family. Most of the so-called specificity groups, which are distinguished on the basis of the preferential binding of lectins to monosaccharides, are represented in the legume lectin family. Only mannose-binding and mannose/maltose- specific lectins have not been identified (yet) in legumes. It is also worth noting that fucose-specific and mannose/glucose-binding plant lectins have only been found in legumes. Moreover, almost all lectins with a so-called ‘complex’ specificity are typical legume lectins. The broad specificity range is at first sight difficult to reconcile with the high sequence similarities and the conserved structure of the legume lectin monomers. Recent studies have demonstrated, however, that substitutions of a few amino acids (which are involved in sugar binding activity) and variations in the length of a particular loop profoundly change the structure of the binding site without affecting the overall three-dimensional structure of the protomer [38, 39]. Most probably, the legume lectin family is the result of an evolution toward a diversity of carbohydrate-binding specificities. The question remains what was and possibly still is the driving force behind this evolution.

2.2.3 Physiological Role

Although the physiological role of legume lectins has been an important issue in lectin research for decades, the possible function of legume lectins is still unclear. Many efforts have been undertaken to prove the hypothesis that legume lectins are responsible for the specific interaction between legumes and their symbiotic nitrogen-fixing *Rhizobia* [40]. However, until now there was no conclusive evidence that legume lectins play a determining role in this process. On the contrary, the discovery that the host specificity of *Rhizobium* strains depends on the structure of the nod factors (also called lipochitin oligosaccharides) they secrete almost excludes an involvement of the legume lectins, as they simply do not have the right specificity. Moreover, the presumed role of the legume lectins in *Rhizobium*-legume symbiosis is even more questioned by the recent discovery of a novel type of carbohydrate-binding protein with a high affinity for Nod factors in the roots of *Dolichos biflorus* [41].

Two major observations argue against the involvement of legume lectins in specific recognition processes within the plant. First, many legume lectins are so abundant that the plant cannot contain a correspondingly high level of specific receptors. Second, most legume lectins have a preferential specificity for carbohydrates that are absent in plants but are important components of animal glycoconjugates (e.g., sialic acid, GalNAc, some complex-type N-glycans) [42, 43]. The obvious abundance taken together with the fact that the lectins from seeds and vegetative storage tissues behave as typical storage proteins for what concerns their cellular and intracellular location, as well as the spatial and temporal regulation of their expression, strongly suggest that legume lectins have a transient storage function. To explain the preferential specificity toward typical animal glycans, the hypothesis has been put forward that legume lectins play a role in the plant's defense against insects and/or predating animals [43, 44]. According to the currently accepted ideas, dietary lectins, which are usually highly resistant to gut proteases, bind to glycan receptors exposed on the surface of the epithelial cells along the gastrointestinal tract of the plant-eating organisms. After binding and possible endocytosis, the lectins elicit specific reactions in the target cells that eventually may result in a noxious effect [45]. For example, ingestion of high doses of PHA causes

acute nausea followed by vomiting and diarrhoea [43, 44]. The eventual discomfort is so severe that experimental animals refuse to continue eating a PHA-containing diet. Although some older reports also claim that PHA is toxic to insects, Murdock et al. [46] have clearly shown that PHA is not toxic to cowpea weevil (*Callosobruchus maculatus*), and the previous toxic effects attributed to PHA are due to the contamination of the lectin with the α-amylase inhibitor. Some other legume lectins are toxic to insects or interfere with insect development when tested *in vitro*. For example, the lectin from the seeds of peanut has an inhibitory effect on the development of cowpea weevil larvae [46]. Similarly, the *Bauhinia purpurea* lectin is lethal to neonate larvae of *Ostrinia nubilalis* and inhibits the growth of *Diabrotica undecimpunctata* larvae [47]. Tests with transgenic plants have shown that tobacco plants expressing the pea lectin gene have increased resistance to *Heliothis virescens* [48]. It should be emphasized, however, that the lectin-related proteins are more potent anti-insect proteins than the legume lectins themselves. For example, low levels of dietary *Phaseolus vulgaris* α-amylase inhibitor effectively inhibit larval growth of typical seed predating insects such as the cowpea weevil (*Callosobruchus maculatus*) and the Azuki bean weevil (*Callosobruchus chinensis*) [49]. Moreover, transgenic pea seeds expressing the bean α-amylase inhibitor acquire resistance against the cowpea and Azuki bean weevil [50].

Summarizing, it can be concluded that legume lectins have a dual role. Under normal conditions they are genuine storage proteins. However, as soon as the plant is eaten the lectins end up in the gastrointestinal tract of the predator and act as defense proteins.

2.3 Separation Techniques

2.3.1 Affinity chromatography

Affinity chromatography separates proteins on the basis of a reversible interaction between protein or group of proteins and a specific ligand coupled to a chromatography matrix. The technique offers high selectivity, hence high resolution, and usually high capacity for the protein(s) of interest. Purification can be in the order

of several thousand-fold and recoveries of active material are generally very high [51].

Affinity chromatography is unique in purification technology since it is the only technique that enables the purification of a biomolecule on the basis of its biological function or individual chemical structure. Purification that would otherwise be time-consuming, difficult or even impossible using other techniques can often be easily achieved with affinity chromatography. The technique can be used to separate active biomolecules from denatured or functionally different forms, to isolate pure substances present at low concentration in large volumes of crude sample and also to remove specific contaminants.

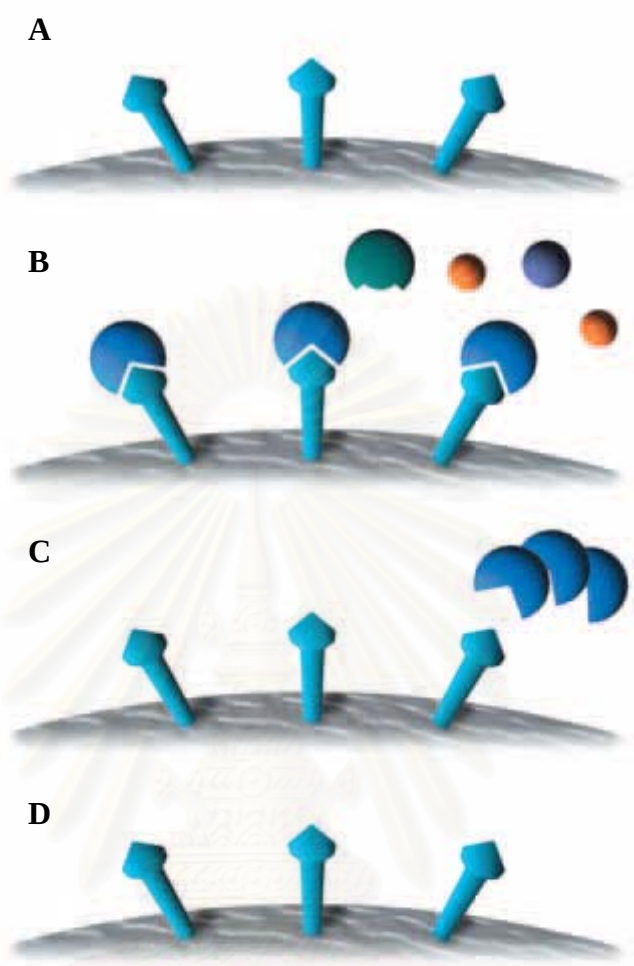


Figure 2.3 A) Affinity medium is equilibrated in binding buffer. B) Sample is applied under conditions that favor specific binding of the target molecule(s) to a complementary binding substance (the ligand). Target substances bind specifically, but reversibly, to the ligand and unbound material washes through the column. C) Target protein is recovered by changing conditions to favor elution of the bound molecules. Elution is performed specifically, using a competitive ligand, or non-specifically, by changing the pH, ionic strength or polarity. Target protein is collected in a purified, concentrated form. D) Affinity medium is re-equilibrated with binding buffer [51].

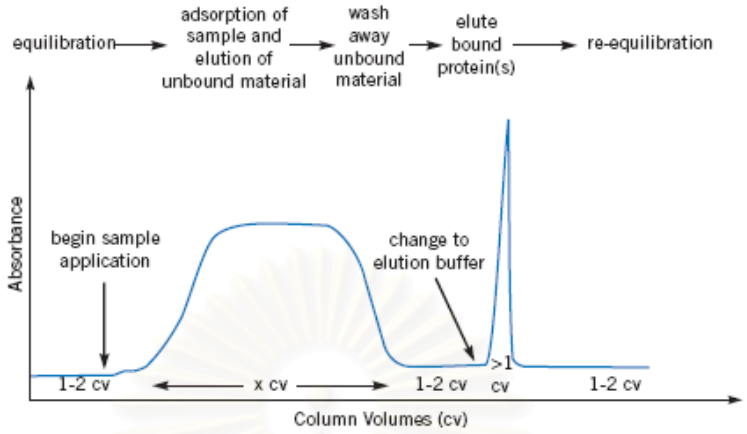


Figure 2.4 Affinity chromatogram [51]

Application for lectins

Some proteins extracted from certain seeds are capable of binding compounds containing carbohydrate groups. These proteins are known as phytohemagglutinins or lectins. Affinity chromatographic media using such lectins have been used to investigate cell membrane structures and aid in the study of cell interactions. They are also used in conjunction with quantitative column chromatographic methods and in some electrophoretic separations of carbohydrate-rich proteins [52].

Concanavalin A

ConA Sepharose™ is Concanavalin A coupled to Sepharose 4B by the cyanogens bromide method.

Concanavalin A (ConA) is a tetrameric metalloprotein isolated from *Canavalia ensiformis* (jack bean). ConA binds molecules containing α-D-mannopyranosyl, α-D-glucopyranosyl and sterically related residues. The binding sugar requires the presence of C-3, C-4 and C-5 hydroxyl groups for ratio with ConA. ConA coupled to Sepharose is routinely used for separation and purification of glycoproteins, polysaccharides and glycolipids. Other application areas where ConA Sepharose 4B has been used are purification of enzyme-antibody conjugates,

purification of IgM, isolation of cell surface glycoproteins from detergent-solubilized membranes [53].

2.3.2 Sodium dodecyl sulfate polyacrylamide gel electrophoresis

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) is the most widely used method for analyzing protein mixtures qualitatively. It is particularly useful for monitoring protein purification and, because the method is based on the separation of proteins according to size. The method can also be used to determine the relative molecular mass of proteins. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) in the presence of a reducing agent (2-mercaptoethanol) is a technique for the separation of polypeptide subunits according to their molecular weight. The protocol involves denaturing the protein sample by heating it in the presence of SDS and a reducing agent. SDS will bind to the protein causing it to unfold, whereas the reducing agent will reduce the intramolecular and intermolecular disulfide bonds. The binding of SDS by the protein confers a net negative charge and the denatured polypeptide will migrate through a gel of known percent acrylamide in the presence of an applied electric field towards the positive electrode (anode). After the electrophoresis is complete, the gel is stained with Coomassie" Blue R-250 to visualize the polypeptide bands. The molecular weight of the polypeptide is inversely proportional to its mobility. The molecular weight of the polypeptide subunit can be estimated directly from a semilog graph of the molecular weight of standard proteins versus their mobility or from a plot of the log of molecular weight versus mobility. Separation of proteins by SDS-PAGE is an excellent technique for producing individually "purified" proteins [54].

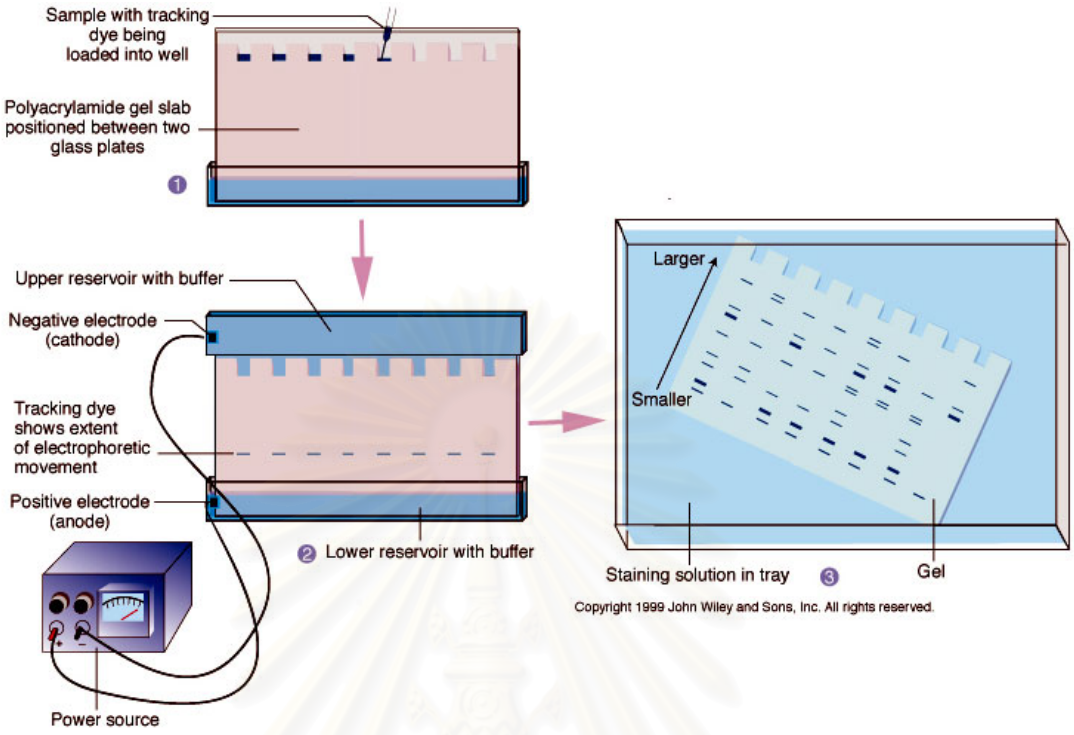


Figure 2.5 SDS-PAGE [54]

One-dimensional gel electrophoresis

The one-dimensional gel electrophoresis has two parts. The first part is stacking gel. The stacking gel is used to concentrate sample proteins in order to be a sharp band before it goes to the second part or separating gel. The stacking gel has a large pore size, which the proteins can move freely under the electric field. Then negatively charged protein-SDS complexes continue to move towards the anode through the second part which is separating gel. The smaller proteins are more easily pass though the pore of gel, whereas large proteins are successively retarded by frictional resistance due to the sieving effect of gel [54].

2.4 Protein Identification Techniques

2.4.1 Mass spectrometry

Mass spectrometry (MS) is an analytical technique for the determination of the elemental composition of a sample or molecule. It is also used for elucidating the chemical structures of molecules, such as peptides and other chemical compounds. The MS principle consists of ionizing chemical compounds to generate charged molecules or molecule fragments and measurement of their mass-to-charge ratios. In a typical MS procedure, a sample is loaded onto the MS instrument, and its compounds are ionized by different methods (e.g., by impacting them with an electron beam), resulting in the formation of charged particles (ions). Then the generated ions are calculated their mass in mass analyzer component and detector [55].

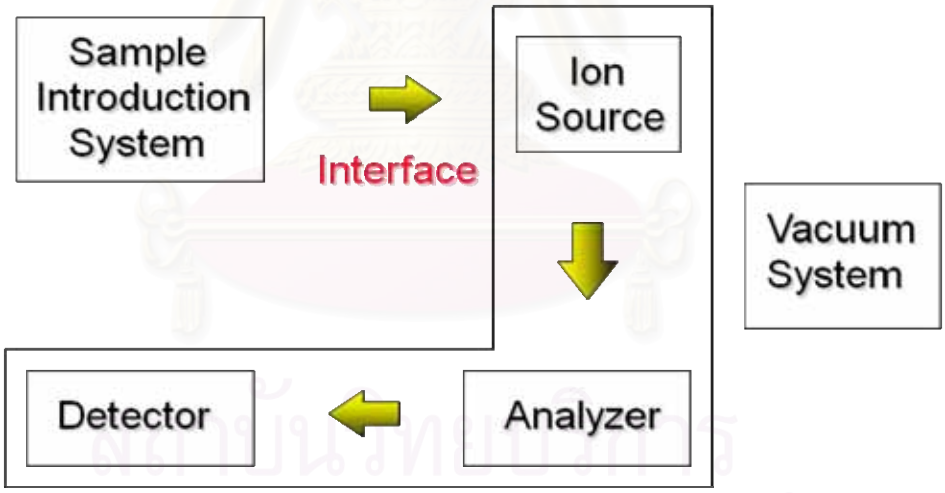


Figure 2.6 A full diagram of a mass spectrometer [55].

2.4.1.1 Electrospray Ionization

Electrospray Ionization (ESI) is one of the Atmospheric Pressure Ionization (API) techniques and is well-suited to the analysis of polar molecules ranging from less than 100 Da to more than 1,000,000 Da in molecular mass [55].

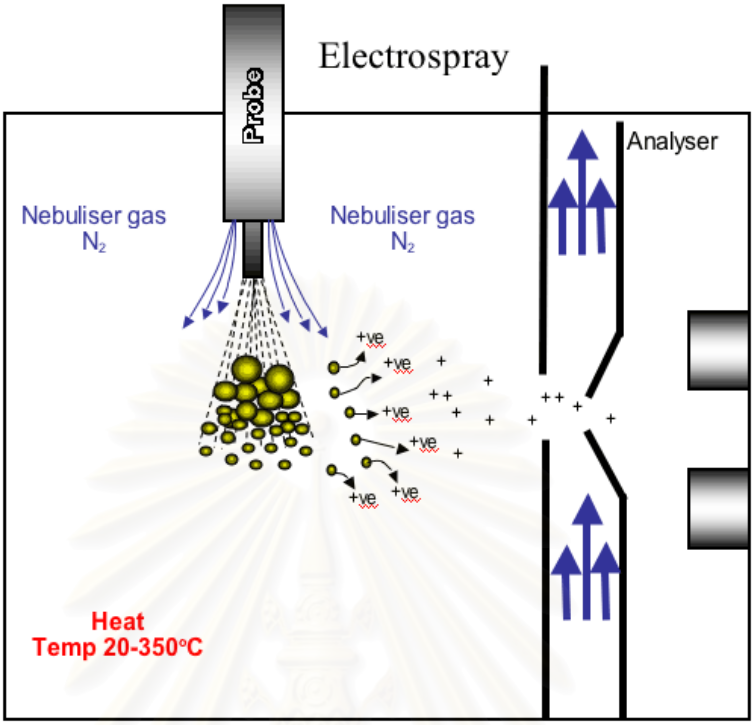


Figure 2.7 Electrospray ionization source [55]

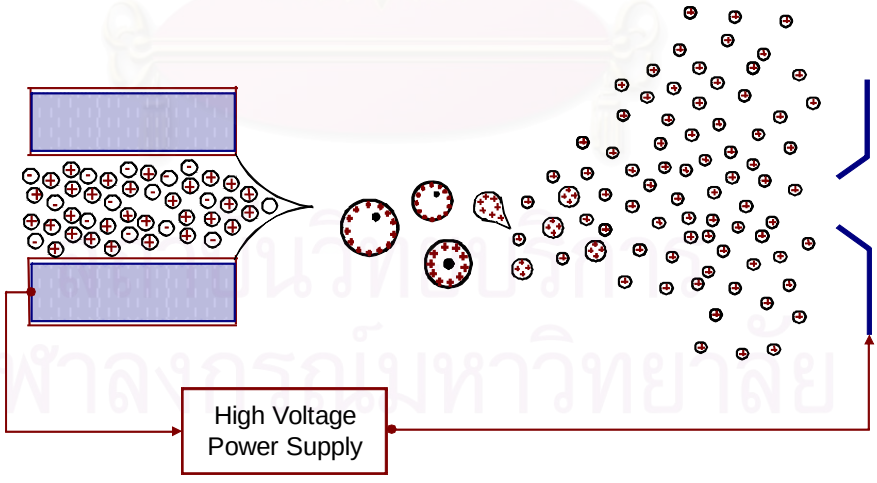


Figure 2.8 The electrospray ionization process [55]

A fundamental challenge to the application of mass spectrometry to any class of analytes is the production of gas-phase ions of those species, and difficulties in producing gas-phase ions can prevent mass spectrometric analysis of certain classes of molecules. This situation was once the case with protein and peptides. The first techniques that were applied, electron ionization and chemical ionization, are two step processes in which the analyte is vaporized with heat and ionization occurs once the analyte is in the gas phase. This vaporization step limited mass spectrometric sequencing experiments to the analysis of small peptides, usually to a maximum of 4 to 5 amino acids. Further, these peptides had to be derivatized to minimize polarity and to give them sufficient volatility. The analysis of proteins was simply not possible, and similar problems were encountered with other classes of polar molecules.

The ionization methods most commonly used to volatized and ionize the proteins or peptides for mass spectrometry is Matrix-Assisted Laser Desorption/ionization (MALDI)

2.4.1.2 Matrix assisted laser desorption ionization

MALDI is based on the bombardment of sample molecules with a laser light to bring about sample ionization. The sample is pre-mixed with a highly absorbing matrix compound for the most consistent and reliable results. The matrix transforms the laser energy into excitation energy for the sample, which leads to sputtering of analyte and matrix ions from the surface of the mixture. In this way energy transfer is efficient and also the analyte molecules are spared excessive direct energy that may otherwise cause decomposition. Most commercially available MALDI mass spectrometers now have a pulsed nitrogen laser of wavelength 337 nm [56].

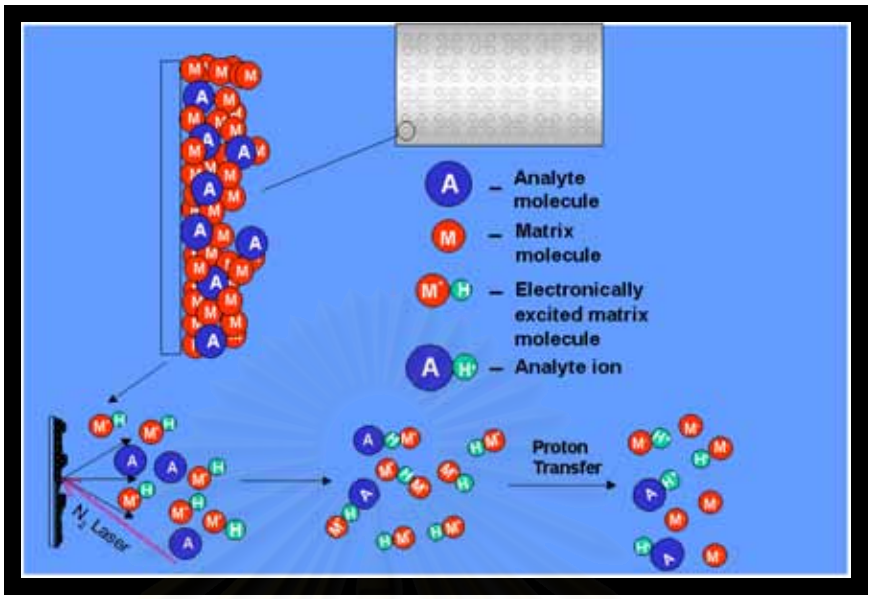


Figure 2.9 Matrix assisted laser desorption ionization (MALDI) [56]

2.5 Protein identification by database searching

The Protein Sequence Databases [57]

Protein identification is made by comparing the mass spectrometric data with the information that has been gathered into the sequence databases.

- SWISS-PROT (www.expasy.ch/sprot-top.html) is a database of annotated protein sequence; it also contains addition information on function of the protein, its domain structure, posttranslational modification, etc.

- TrEMBL (www.expasy.ch/srs57) is a supplement to SWISS-PROT, which contains all protein sequences, translated from nucleotide sequences of the EMBL database.

- NCBInr (www.ncbi.nLm.nilygov/dbEST/) is a database containing sequences translated from DNA sequences of GenBank and also sequences from PDB, SWISS-PROT and PI database.

2.6 Literature review

Parkia speciosa seed lectin was purified from a crude extract by $(NH_4)_2SO_4$ fraction followed by specific adsorption on Sephadex G-100 and subsequent displacement with D-glucose. The molecular weight of purified lectin determined by gel filtration was 46.7 and 47.3 kDa by denaturating gel electrophoresis. The lectins agglutinated rat erythrocytes but not those of humans. Sugars which specifically bind with the lectin were tested by hemagglutination inhibition with D-glucose, D-manose, D-fructose, maltose, *N*-acetyl-D-glucosamine, α-methyl-D-glucopyranoside and methyl-α-D-mannopyranoside. Amino acid compositions of lectin are glycine, aspartic acid, isoleucine, serine, threonine and the amount (mol %) of these amino acids are 15.8, 11.3, 9.3, 9.2 and 7.1, respectively [58].

A new lectin was purified from the seeds of *Butea monosperma* by affinity chromatography on N-acetyl galactosamine-agarose. The purified lectin has an apparent molecular mass of 67 kDa by gel filtration on a Superose 6HR 10/30 column. The lectin agglutinates human erythrocytes but not those of rat, mouse, hamster, goose and pigeon. The agglutinating activity is inhibited by N-acetyl galactosamine and does not require a divalent ion. It is also stable at up to 80°C for 60 min [59].

A glycoprotein capable of binding simple carbohydrates and causing hemagglutination has been isolated from seeds of the legume plant sainfoin (*Onobrychis viciifolia*, Scop. var Eski). The phytolectin was prepared by affinity chromatography of pH 7.0 sodium phosphate extracts on columns of Sepharose-4B containing covalently attached D-mannose. Molecular weight determinations showed the lectin to be a dimer consisting of 26 kDa, non-covalently associated monomers. Carbohydrate-binding specificity was directed toward D-mannose and D-glucose and their α-glycosidic derivatives. The purified protein agglutinated cat erythrocytes at 5 µg/ml [60].

A new galactose-specific lectin was isolated from African yam bean (*Sphenostyles stenocarpa* Harms) by affinity chromatography on galactose-Sepharose 4B. SDS-PAGE analysis resulted in four polypeptide bands of approximately 27, 29, 32 and 34 kDa, respectively. Based on the analysis of carbohydrate content and native PAGE, it is likely that the *Sphenostyles* lectin is a tetrameric glycoprotein with MW of approximately 122 kDa. N-terminal protein sequencing of purified lectins from four different *Sphenostyles* accessions shows that the four polypeptides have largely identical amino acid sequences. The sequences contain the conserved consensus sequence F-F-LILG characteristic of legume lectins, as well as *Phaseolus vulgaris* proteins in the arcelin-α-amylase inhibitor gene family. The lectin agglutinates both rabbit and human erythrocytes, but with a preference for blood types A and O [61].

CHAPTER III

EXPERIMENTAL

3.1 Biological materials

Seeds of *A. jiringa* purchased from the local market (Bangkok, Thailand). A voucher specimen (BK8261) is deposited at the Bangkok Herbarium of The Plant Variety Protection Division, Department of Agriculture, Bangkok, Thailand. The human blood was obtained from the blood donation office of The Thai Red Cross Society, Bangkok, Thailand. Whereas a variety of animal blood was supplied from the Division of Production and Supply, National Laboratory Animal Center, Mahidol University, Nakhon Pathom, Thailand. Strain of fungal pathogen; *Colectrotrichum cassicola*, *Exserohilum turcicum,* and *Fusarium oxysporum* were provide from the Division of Plant Disease and Microbiology, Department of Agriculture, Bangkok, Thailand.

3.2 Chemicals and reagents

Concanavalin A (Con A) was purchased from Sigma Chemicals Co. (St. Louis, MO, USA). Methyl-α-D-glucopyranoside and EDTA were purchased from Fluka (Germany). The reagent and staining kit used in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) were obtained from Plusone Pharmacia Biotech (Uppsala, Sweden). The low molecular weight calibration kit was used as standard molecule weight marker proteins and purchased from Amersham Pharmacia biotech (UK). Brilliant Blue G, Bovine serum albumin and p-nitrophenol 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 sulfate $(NH_4)_2S0_4$, Calcium chloride, Mercury chloride, Sodium acetate, Sodium hydroxide and Sodium chloride (NaCl) were obtained from Merck (Germany). Cobalt chloride, Copper sulfate, Dimethyl sulfoxide, D-Glucose, Lactose, Maltose and Manganese Chloride were obtained from Fluka (Switzerland). Tris was purchased from USB (USA). Methanol (MeOH), Ethanol and Acetic acid were purchased from Merck

(Germany). Double distilled water was used in this research was prepared with Glass water Sills (GFL Gesellschaft fur labortecilk mbH, Germany).

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) 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, HITACHI, Japan) Water Bath Shaking (Memmert, Germany) Power Supply (EPS 3500 XL, Pharmacia, England) Dialysis bag (Rockford, USA), Autoclave (Isuzu, Seisakushu Co., Ltd., Japan) Freeze dryer (Labconco, USA) Microcentrifuge (Biofuge pico Heraeus, Kendro, Germany) Spectrophotometer (TECAN, Austria).

3.4 Procedures

3.4.1 Extraction of *A. jiringa* seeds lectin

One kilogram of the *A. jiringa* seeds was defatted with acetone at 4^{o}C and extracted overnight at 4^{o}C with 20 mM Tris-HCl, pH 7.2 containing 0.15 M NaCl. After centrifuged (10,000 rpm, 15 min, 4^{o}C), protein in the supernatant was precipitated with 90% ammonium sulfate. The protein was dissolved and dialyzed in distilled water then was freezed dry.

3.4.2 Purification of *A. jiringa* seeds lectin

ConA Sepharose was equilibrated with 20 mM Tris-HCl, pH 7.2 containing 150 mM NaCl and transferred to 1.6x20 cm column. The pre-purified extract was applied to the column at flow rate of 1.5 ml/min. The column was washed with the buffer at the same speed until the A_{280} fell down to <0.05. The buffer was exchanged by the eluting buffer (20 mM Tris-HCl, pH 7.2 containing 150 mM NaCl with 0.2 M

Methyl-α-D-glucopyranoside) to desorb the lectin from the column. The fractions containing lectin were combined on the basis of A_{280}, dialyzed against water, frozen and lyophilized.

3.4.3 Protein determination

Protein concentrations in the lectin preparations were determined by the method of Bradford [62] with reference to a standard calibration curve for bovine serum albumin. During the column chromatographic separations the elution profiles of proteins were determined by measuring the absorbance at 280 nm.

3.4.4 Hemagglutination assays

Serial twofold dilutions of purified lectin 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 scored after 1 h at room temperature. 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, rat, mouse, guinea pig, goose, sheep and human ABO erythrocytes.

3.4.5 SDS polyacrylamide gel electrophoresis

The gel was prepared with 0.1% SDS in 12.5% separating gels and 5.0% 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 [63]. Samples to be analyzed were treated with sample buffer and boiled for 5 min prior to application to the gel. Electrophoresis was run from the cathode to anode at a constant current of 20 mA per slab at room temperature in a Mini-Gel Electrophoresis unit. Molecular weight standard was used to determine the subunit molecular weight of the lectin. After electrophoresis, proteins in the gel were visualized by staining with Coomassie blue R-250.

3.4.6 Effect of temperature on lectin hemagglutinating activity and thermostability

The effect of temperature on lectin hemagglutinating activity was determined by incubating lectin samples (pH 7.2) at various temperatures (30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90 and 95^{o}C) at room temperature for 30 min. Thermostability was investigated by incubating lectin samples at 40, 50, 60 and 70^{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.

3.4.7 pH-dependence of agglutination activity

The effect of pH on lectin hemagglutinating activity was determined by incubating lectin samples at various pHs (2-12) at room temperature for 1 h. The following buffers were used: 0.1 M glycine-HCl buffer (pH 2.0-3.0); 0.1 M Sodium acetate buffer (pH 4.0-5.0); 0.1 M Potassium hydrogen phosphate buffer (pH 6.0-7.0); 0.1 M Tris-HCl buffer (pH 8.0-9.0) and 0.1 M glycine-NaOH buffer (pH 10.0-12.0). The residual hemagglutinating activity was assayed after adjusting the mixture to pH 7.2. At least three replicates were done for each test to confirm the results.

3.4.8 Effect of metal ions

The purified lectin (1 mg/ml) was incubated for 10 h with Ca^{2+}, Co^{2+}, EDTA, Hg^{2+}, Mg^{2+}, Mn^{2+} and Fe^{2+} at various concentrations with continuous shaking. After that, 50 µl of a 2-4% suspension of rabbit erythrocytes were added, and the hemagglutination was scored after 1 h.

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

The internal amino acid sequence of the purified lectin from *A. jiringa* seeds 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.

3.4.10 Assay of antifungal activity

The antifungal activity 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) 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 25°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. The fungal species used included *E. turicicum*, *F. oxysporum*, and *C. cassiicola*. Petri dish was incubated at 25^{o}C for 5 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.

3.4.11 Assay for alpha glucosidase inhibitor

α-Glucosidase activity assay was carried out according to Adisakwattana *et al* [64]. α-Glucosidase activity was assayed using 50 mM acetate buffer at pH 4.5. The lectin sample at the designated concentration was premixed with 50 µl enzyme solution (1 U/ml) and incubated at 37 °C for 10 min. Then 950 µl of 1 mM PNPG as a substrate was added to the mixture to initiate the enzyme reaction. The reaction was incubated at 37 °C for 20 min and stopped by adding 1 mL of 1 M Na_2CO_3. α-Glucosidase activity was determined by measuring release of the yellow *p*-nitrophenol at 405 nm.

3.4.12 Cytotoxicity test against cancer cell lines

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) was investigated. These cells were trypsinized before seeding at a density of 1×10^4 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.

CHAPTER IV

RESULTS AND DISCUSSION

The lectin from *A. jiringa* was analyzed using all methods as described in the chapter III. The results have been shown and discussed in each part of this chapter, respectively.

4.1 Purification of lectin from seeds of *A. jiringa*

A lectin from seeds extract of *A. jiringa* was purified in a single step by affinity chromatography column using ConA Sepharose to isolate lectin in milligram amounts. The purification protocol of this lectin is summarized in Table 4.1. The unretained fraction was eluted with selected buffer, and showed no hemagglutination activity in the presence of any tested erythrocytes. The chromatographic profile revealed a protein peak eluted with 0.2 M Methyl-α-D-glucopyranoside. Highest hemagglutination was detected only on fractions obtained after elution, and led to increments in the specific activity up to 88.27 $x10^2$ HU/mg corresponding to 51.61% yield (Figure 4.1). The lectin bound irreversibly to ConA Sepharose and displacement of the bound protein was difficult. Hydrophobic interactions may be responsible for the poor elution with Methyl-α-D-glucopyranoside. Secondary non-specific polar interactions along with specific ConA glycoprotein interactions may be playing a role in the binding of lectin to the affinity matrix [65]. The lectin yield obtained from this study is quite high when compared with previous lectin purification from the seeds of *Mimosa invisa* L. which was also purified by affinity chromatography column using ConA Sepharose [66].

Table 4.1 Purification table of the lectin form seeds of *A. jiringa*.

Purification step	**Total protein (mg)[a]**	**Total lectin (titer x ml)[b]**	**Specific activity (HU/mg)[c]**	**Yield (%)**	**Purification (fold)[d]**
Crude extract	47.58	2.71×10^4	5.69×10^2	100.00	1.00
90 % $(NH_4)_2SO_4$ precipitation	7.11	1.79×10^4	25.19×10^2	66.16	4.43
ConA Sepharose[e]	1.58	1.40×10^4	88.27×10^2	51.61	15.51

[a] Crude protein extract from 200 g of cotyledons.

[b] Minimal concentration of protein able to cause visible agglutination of a 2-4% 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.

[e] The lectin was obtained by affinity chromatography on ConA Sepharose.

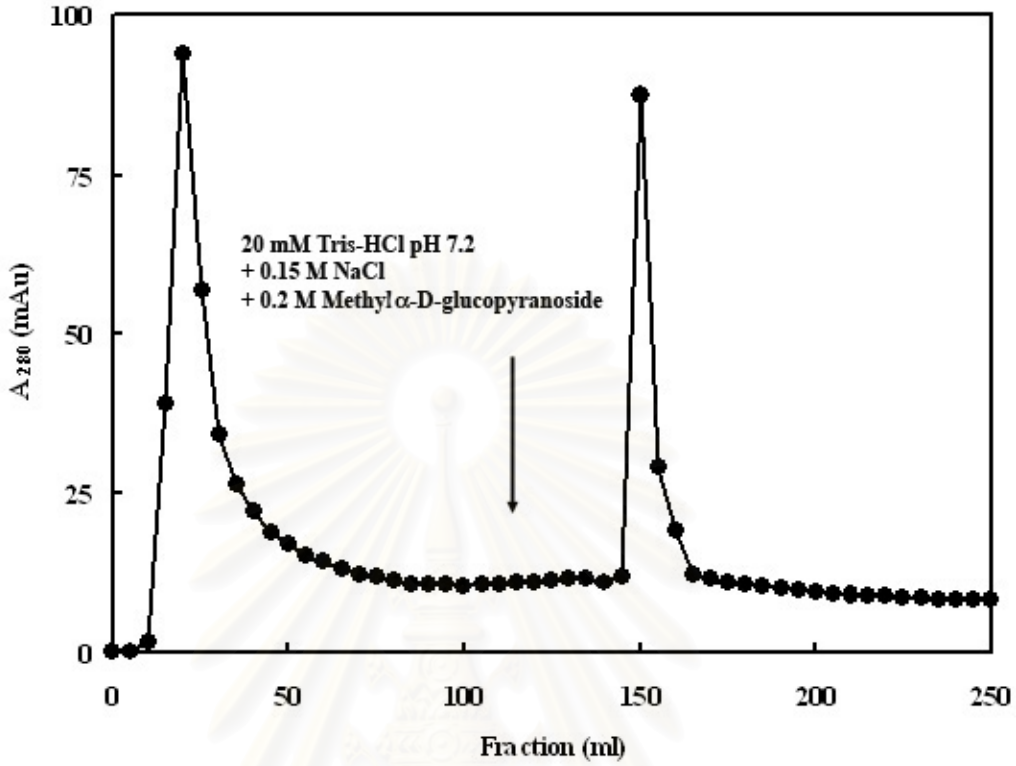


Figure 4.1 Affinity chromatogram of lectin on ConA Sepharose column (1.6x20 cm) equilibrated with 20 mM Tris-HCl pH 7.2 containing 0.15 M NaCl. Lectin was eluted with 20 mM Tris-HCl pH 7.2 containing 0.15 M NaCl, 0.2 M Methyl-α-D-glucopyranoside at a flow rate of 1.5 ml/min.

4.2 SDS-PAGE analysis

Polyacrylamide gel electrophoresis of purified *A. jiringa* lectin preparation, treated with SDS for protein, showed to be a sensitive technique. The mobility of a macromolecule through a gel under an electric field depends upon its charge on its molecular mass, size and shape [67]. SDS-PAGE analysis of the purified lectin from seeds of *A. jiringa* showed the presence of a single band when stained with Coomassie blue R-250 and this was about 35.7 kDa (Figure 4.2). From, previously publication on biochemical characteristics of the other plant lectin, the molecular mass of lectin from other plants ranges from 30 to 50 kDa [68, 69, 70].

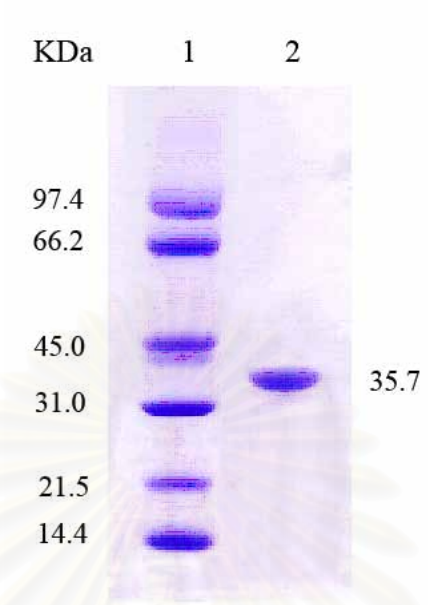


Figure 4.2 SDS-PAGE analysis of lectin. Lane 1, molecular weight standards; Lane 2 purified lectin from seeds of *A. jiringa*.

4.3 Assay for Hemagglutinating activity

A. jiringa lectin showed no specificity in its ability to hemagglutinate human (A, B, AB and O) erythrocytes and indiscriminately agglutinate rabbit, rat, mouse, guinea pig, goose and sheep. However, hemagglutinating activity against mouse and guinea pig erythrocytes were comparatively the lowest ones (Table 4.2). In this respect it is similar to other previous studies of a lectin from Egyptian *P. sativum* seeds [69] and *Bauhinia monandra* [71]. Several lectins demonstrate a preference in agglutinating one more type of human or of a certain animal erythocytes as the *Sphenostyles stenocarpa* lectin that demonstrated high agglutination of human blood type O [61], the *Hevea brasiliensis* lectin that preferentially agglutinated rabbit erythrocytes [72], and *Talisia esculenta* lectin that demonstrated preference for human blood type AB [73]. The classical and still simplest way to detect the presence of a lectin in material biological is to prepare an extract from material and examine its ability to agglutinate erythrocyte [8]. For agglutination to occur, the lectin must bind to the cells and from cross-bridge between them. There is however no simple relation between the amount of lectin bound and agglutination. This is because agglutination

is affect by many factors among them accessibility of receptor sites and also influence by external conditions of the temperature, cell concentration, and mixing. This difference in the agglutination activity may be due to the nature of the glycoproteins protruding on the cell surface, which are weakly or not totally recognized by the lectin [74].

Table 4.2 Hemagglutinating activity of *A. jiringa* lectin against human and animal erythrocytes

Erythrocyte sources	Agglutination (titer)[a]
Mouse	2^3
Rat	2^4
Guinea pig	2^3
Goose	2^4
Sheep	2^5
Rabbit	2^4
Human Type A	2^4
Human Type B	2^4
Human Type O	2^5
Human Type AB	2^4

[a] Titer is defined as the reciprocal of the end point dilution causing detectable agglutination of erythrocytes. The initial amount of *A. jiringa* lectin used in these assays was 99.47 µg and diluted by 1/2 for the subsequent serial dilutions.

4.4 Effect of temperature on lectin activity and stability

The hemagglutinating activity of *A. jiringa* is still fully active at the temperature below 45^{o}C for 30 min. At temperature above 45^{o}C, its activity was dropped gradually to 50, 25, 25, 12.5 and then 6.25% when the temperature was increased to 50, 55, 60, 65 and 70^{o}C, respectively. It is completely inactive at 75^{o}C for less than 30 min (Figure 4.3A). The maximum activity temperature was below 45^{o}C, while 50% of its maximum activity retained after 120 min of incubation at 40^{o}C. The activity was rapidly inactivated at 70^{o}C (Figure 4.3B). The thermostable lectin which has been reported is from *Momordica charantia* [75]. It's active up to 55 °C. The loss of hemagglutinating activity with increasing temperature is evidently due to denaturation. This denaturation may expectedly weaken the interaction between lectin and the carbohydrate ligand leading consequently to attenuated agglutination activity.

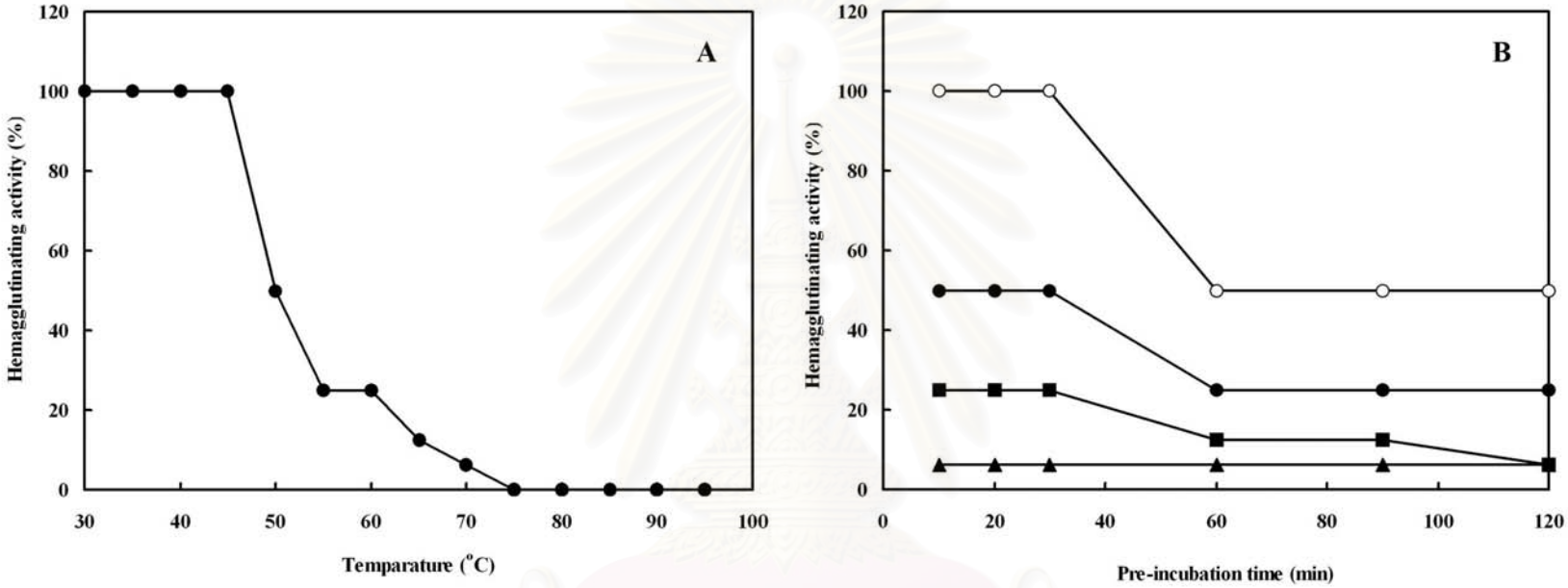


Figure 4.3 A) Effect of temperature on the agglutinating activity of lectin towards rabbit erythrocyte suspension. The 50 mM Tris-HCl, pH 7.2 buffer system was used. B) Thermostability of purified lectin towards rabbit erythrocyte suspension. The 20 mM Tris-HCl, pH 7.2 buffer system was used: (○) 40°C; (●) 50°C; (■) 60°C; and (▲) 70°C. Each point on the line represents the average of three replicates. Full activity (100%) corresponded to a titer of 2^4.

4.5 Effect of pH on lectin activity

Hemagglutination was markedly affected by basic pH, while it maintained 200% of its activity at a pH 8. When decreased from pH 7 to 2, it led to decrease the activity by 100%. On the other hand, when increased the pH above 8, it gradually decreased hemagglutinating activity (Figure 4.4) from 100%, 50%, 3.12% and 1.56% at the pH values 9, 10, 11 and 12, respectively. This behavior has been observed in other lectins, as demonstrated by *Salvia bogotensis* seeds lectin where maximum pH stability was reached at pH 7.0 and 8.0 but its activity below pH 2.0 and above 12.5 was completely inhibited [76]. The relatively lower reduction of activity at the basic pH values may be due to some degree of base-induced denaturation.

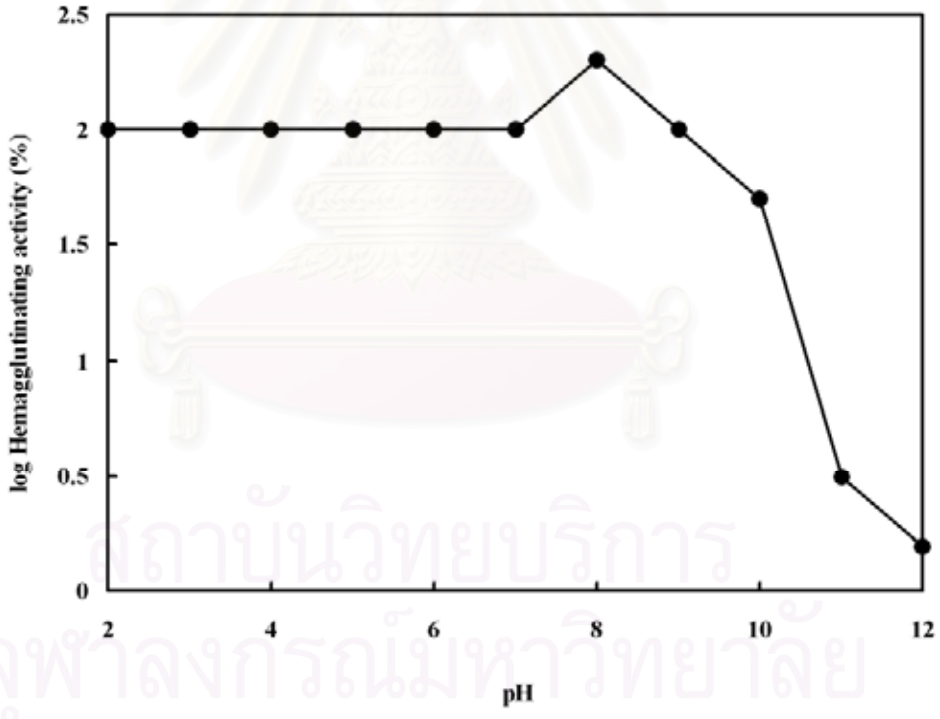


Figure 4.4 Effect of pH on the agglutinating activity of lectin towards rabbit erythrocyte suspension. The 20 mM Tris-HCl, pH 7.2 buffer system was used. Each point on the line represents the average of three replicates. Full activity (100%) corresponded to a titer of 2^7.

4.6 Effect of metal ions

The hemagglutination activity of *A. jiringa* lectin with various metal ions was studied. Ca^{2+}, Mg^{2+}, and Mn^{2+} were absolutely required for hemagglutination activity (Table 4.3). Similar results were observed for *Dioclea altissima* lectin [77]. The requirement for metal is a general physico-chemical property of most legume lectins [31, 78], suggesting that certain divalent cations are essential for the hemagglutination activity.

Table 4.3 Effect of some cations on purified lectin from *A. jiringa* seeds.

Concentration of divalent metal cations (mM)	**$CaCl_2$**	**$MgCl_2$**	**$MnCl_2$**	**$CoCl_2$**	**$FeCl_2$**	**$HgCl_2$**	**EDTA**
8.33	√	√	√	x	x	x	x
16.67	√	√	√	x	x	x	x
33.33	√	√	√	x	x	x	x
66.67	√	√	√	x	x	x	x

Note √ hemagglutinating activity x no hemagglutinating activity

4.7 Protein identification by LC-MS/MS

The internal sequence analysis of the purified lectin from *A. jiringa* seeds was obtained by digestion with trypsin and sequenced with LC-MS/MS and this was found to be **VSSDG SPQGS SVGR.** Comparisons were then made to all protein sequences in the SwissProt database using the search protocol BLAST. Using the database search, the tags were identified as parts of a lectin precursor from *Dioclea guianensis* [79] (Figure 4.5). A high degree of internal amino acid sequence identity between lectin from *A. jiringa* seeds suggested that this protein could be a member of mannose-glucose specific lectin family (Figure 4.6).

		5						10								Accession Number
Archidendron jiringa **lectin**		V	S	S	D	G	S	P	Q	G	S	S	V	G	R	
Lectin precursor (*Dioclea guianensis***)**	**69**	V	S	S	D	G	S	P	Q	G	S	S	V	G	R	**82**	**A9J248**
Concanavalin-A precursor (Con A) (*Canavalia gladiata***, Sword bean)**	**70**	V	S	S	N	G	S	P	Q	G	S	S	V	G	R	**83**	**P14894**
Concanavalin-A precursor (Con A) (*Canavalia ensiformis***, Jack bean)**	**70**	V	S	S	N	G	S	P	Q	G	S	S	V	G	R	**83**	**P02866**
Lectin alpha chain (*Dioclea guianensis***)**	**160**	V	S	S	S	G	D	P	Q	G	S	S	V	G	R	**172**	**P81637**
Lectin alpha chain (*Dioclea rostrata***)**	**160**	V	S	S	S	G	D	P	Q	G	N	S	V	G	R	**172**	**P58908**
Lectin alpha chain (*Gatylia floribunda***)**	**159**	V	S	-	N	G	S	P	Q	S	N	S	V	G	R	**171**	**P81517**
Mannose/glucose-specific lectin (*Gatylia mollis* **, Camaratu bean)**	**157**	V	S	-	N	G	S	P	Q	S	N	S	V	G	R	**169**	**P83721**
Mannose-specific lectin (*Chimonanthus praecox***)**	**102**	Y	S	S	Q	G	S	A	I	W	S	S	K	T	W	**116**	**A2SVT1**

Figure 4.5 Amino acid sequences from the fragment obtained by tryptic digestion of the purified lectin from *A. jiringa* seeds. Comparisons are made with other lectin classified as mannose-glucose specific lectin family. Shaded regions represent regions of identity.

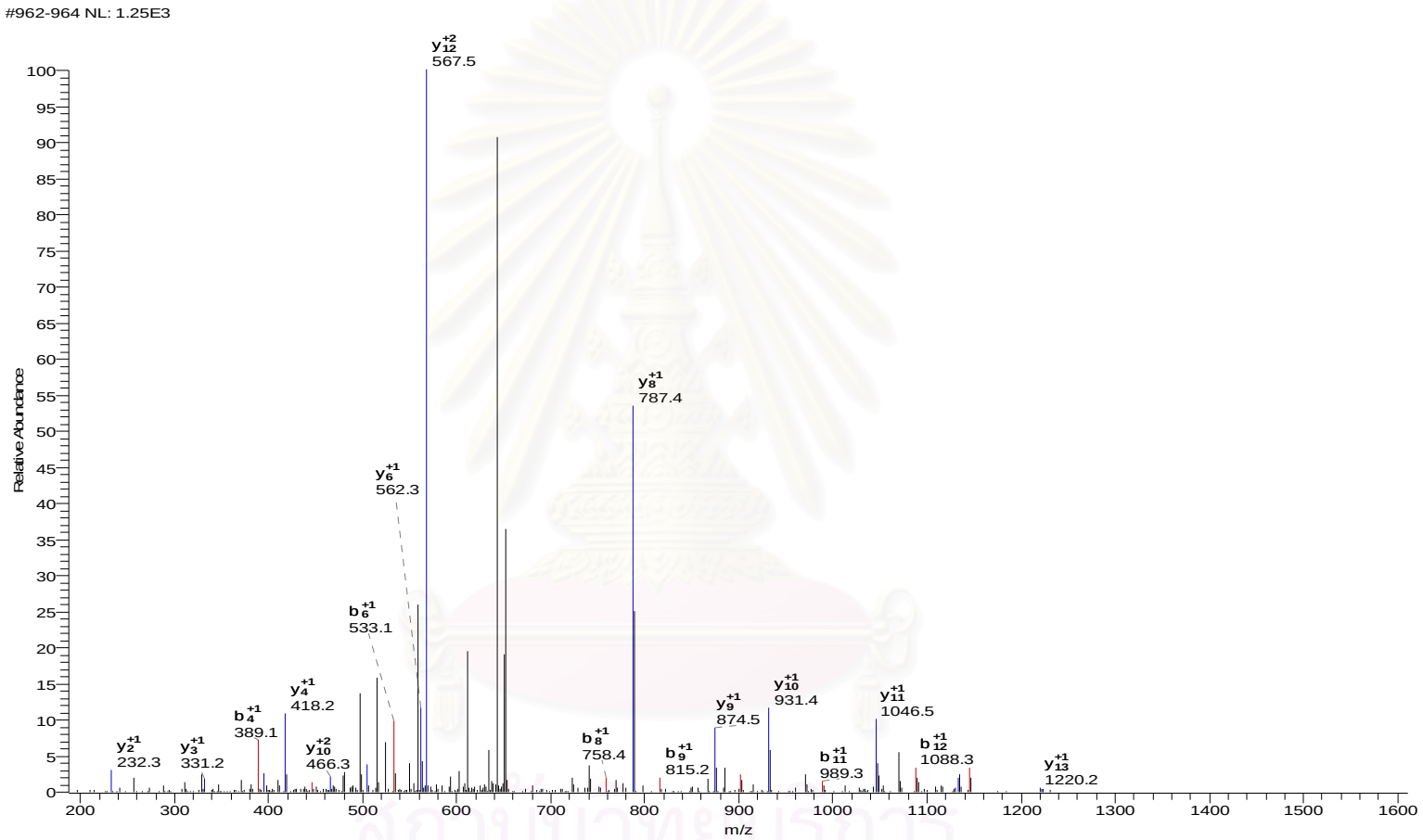


Figure 4.6 MS-MS spectra of purified lectin from *A. jiringa* seeds.

4.8 Assay of antifungal activity

Growth inhibition of *E. turcicum*, *F. oxysporum* and *C. cassiicola* was noticed in the presence of the purified lectin from *A. jiringa* seeds at two serial dilutions. The lectin inhibited the growth of *E. turcicum*, *F. oxysporum* and *C. cassiicola* of 11.31 and 5.66 µg, respectively. Its activity had strongest effect for *C. cassicola* followed by *E. turcicum* and *F. oxysporum*, respectively (Figure 4.7).

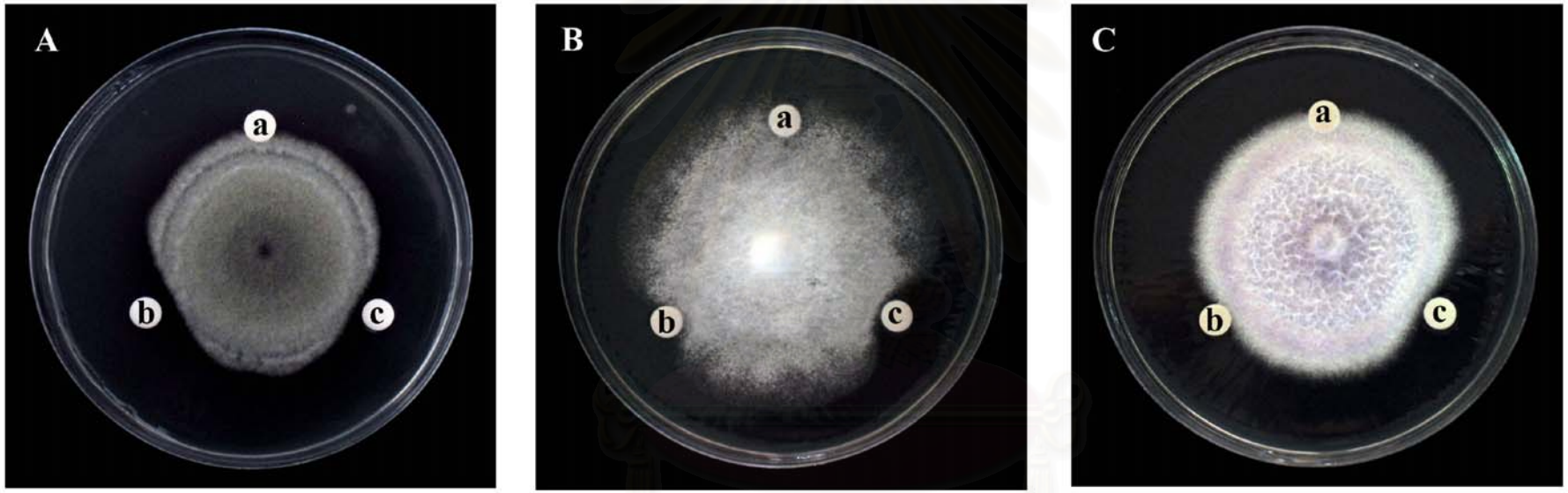


Figure 4.7 Inhibitory effect of *A. jiringa* lectin on antifungal protein toward A) *C. cassiicola*, B) *F. oxysporum*, and C) *E. turicicum*. The negative control is 10 µl of (a) 20 mM Tris-HCl buffer pH 7.2, (b) 5.66 µg *A. jiringa* lectin, and (c) 11.31 µg *A. jiringa* lectin.

The observed antifungal activity of *A. jiringa* against *E. turcicum*, *F. oxysporum* and *C. cassiicola* agrees with the results obtained from other plant legume lectins. This activity was concluded to be related to the lectin carbohydrate binding property, that might endow lectin molecules with binding activity towards certain carbohydrate components in the fungal cell wall affecting its activity and viability as most lectins recognize either N-acetylneuraminic acid, N-acetylglucosamine, N-acetylgalactosamine, galactose, mannose, or fucose in accordance with the conclusion of Lis and Sharon [15]. Many studies of plant lectins have assumed that they are implicated in host defense mechanism as antifungal proteins. However, at the present, only a small number of lectins have been reported to have antifungal activity. The antifungal properties of plant lectins have been found from potato tuber [19], *Amaranthus caudatus* seed [21], stinging nettle rhizomes [22], wheat germ, and recently from *Phaseolus vulgaris* seed [23].

4.9 Assay for alpha glucosidase inhibitor

The amount of the purified lectin from *A. jiringa* seeds used in these assays was 4.97 µg, and led to the percentage of α-glucosidase inhibitory activity at 52.29%. The observed α-glucosidase inhibitor of *A. jiringa* was comparatively higher than the results obtained from other plant in Mimosaceae family [80].

4.10 Cytotoxicity test against cancer cell lines

A. jiringa lectin showed no cytotoxicity for five tested cancer cell lines. The result was similar to other previous studies from Kaowjowjom (*Guaiacum officinale*) and Karawek (*Artabotrys siamensis*) [81]. There were several kinds of plant lectins reported on anti-proliferative effect on tumor cell lines such as *Cratylia mollis* lectin, and *Viscum album* lectin.

CHAPTER V

CONCLUSION

The lectins from *A. jiringa* were studied. The lectins from the seeds of *A. jiringa* were extracted and precipitated by 90% ammonium sulfate ($(NH_4)_2S0_4$) solution. The proteins from this precipitation were not denatured and could be further tested for biological activity.

1. The lectin could be successfully purified in a single step by affinity chromatography.
2. The final yield of lectin was ca. 1.58 mg per 200 g dry seed weight.
3. The molecular mass of purified lectin was 35.7 kDa as estimated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis.
4. The purified lectin showed no specificity in its ability to hemagglutinate human (A, B, AB and O) erythrocytes and indiscriminately agglutinate rabbit, rat, mouse, guinea pig, goose and sheep.
5. Hemagglutination activity of lectin was markedly affected at pH 8. It was heat stable below 45^{o}C for 30 min. The activity was decreased to 50% when heated at 40^{o}C for 120 min and rapidly fully inactivated at 70^{o}C.
6. From the experimental result, it was suggested that *A. jiringa* lectin required divalent metal cations (Ca^{2+}, Mg^{2+}, and Mn^{2+}) for hemagglutination activity.
7. The purified lectin had an internal amino acid sequences composition which is similar to that of mannose-glucose specific lectin family.
8. The purified *A. jiringa* lectin inhibited the growth of *F. oxysporum*, *E. turicicum* and *C. cassiicola* at the concentration of > 5.66 µg.
9. The 4.97 µg of purified lectin led to the 52.29% of α-glucosidase inhibitory activity.
10. *A. jiringa* lectin showed no cytotoxicity for five cell lines.

Suggestion for future work

According to this study, the lectin could be simply extracted and purified at relatively higher yield from *A. jiringa* seeds. It also showed hemagglutination and α-glucosidase inhibition activities. Consequently, this lectin should be studied in more detail using X-ray crystallographic technique. Moreover, α-glucosidase activity should be carried out in detail.

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APPENDICES

APPENDIX A

MEDIA

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 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 and sterilize by autoclaving at 121°C for 15 minutes.

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

12.5% Separating gel

Solution A	4.2 ml
Solution B	2.5 ml
Distilled water	3.3 ml
10% Ammonium persulfate	50 µl
TEMED	5 µl

5.0% Stacking gel

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

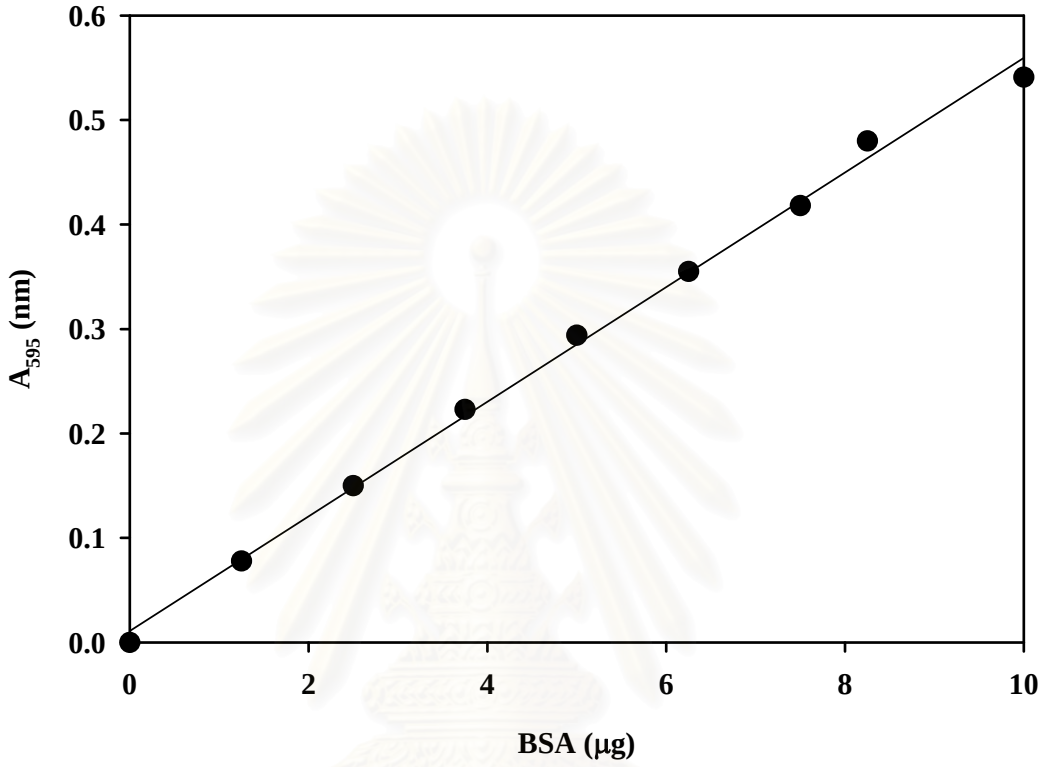
APPENDIX C

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

APPENDIX D

Calibration curve for protein determination Bradford method



VITA

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