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ISOLATION AND CHARACTERIZATION OF EPICUTICULAR AGGLUTININ FROM SEEDLING OF SOYBEAN (*Glycine max*)

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พิมพ์ต้นฉบับบทคัดย่อวิทยานิพนธ์ภายในกรอบสีเขียวนี้เพียงแผ่นเดียว

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ในการศึกษานี้พบว่า มีสารแอกกลูตินิน บนพื้นผิวของกล้าถั่วเหลือง (Glycine max. L.) ซึ่งสามารถสกัดได้โดยการแช่ในน้ำกลั่นแม้ในช่วงเวลาสั้นๆ แค่ 1 นาที แต่จะสกัดได้มากที่สุดถ้าไว้นาน 91 นาที แอกกลูตินินที่สกัดได้ จะพบปริมาณต่างกันในกล้าอายุต่าง ๆ ในกล้าอายุน้อยจะพบสารแอกกลูตินินจากพื้นผิวมากกว่ากลาอายุมากขึ้น สารแอกกลูตินินที่สกัดได้ จะมีสูงสุดบนพื้นผิวของใบเลี้ยงของกล้าถั่วเหลืองอายุ 3 วัน สารแอกกลูตินินที่สกัดได้มีความจำเพาะต่อเม็ดลือดแดงคน กลุ่ม A และเม็ดเลือดแดงกระต่ายที่ถูกย่อยด้วยทริปซินแล้ว แต่ไม่มีความจำเพาะต่อเม็ดเลือดแดงคนกลุ่ม B และ O การตกตะกอนเม็ดเลือดแดงสามารถถูกยับยั้งได้อย่างจำเพาะโดยน้ำตาลกาแลคโตสและอะเซติลกาแลคโตซามีน สารแอกกลูตินินที่สกัดได้ถูกทำให้บริสุทธิ์ โดยผ่านขั้นตอนการตกตะกอนด้วยแอมโมเนียม ซัลเฟต และผ่านแอฟฟินิตี้คอลัมน์ สารที่ได้จากการชะคอลัมน์ด้วยน้ำตาลกาแลคโตสคือสารแอกกลูตินินจากพื้นผิวถั่วเหลือง (SSA : soybean surface agglutinin) เมื่อศึกษาสมบัติของสาร SSA พบว่าให้แอกติวิตี้สูงสุดที่อุณหภูมิ 30-40°C และในบัฟเฟอร์ที่มี pH 8.0 เมื่อศึกษาน้ำหนักโมเลกุลของ SSA ด้วย SDS - โพลีอะคริลาไมด์ เจล อิเลคโตรโฟริซิส และเจลฟิลเตรชั่น พบว่า SSA มีน้ำหนักโมเลกุลของหนึ่งหน่วยย่อยประมาณ 28,000 ดัลตัน ซึ่งก็ใกล้เคียงกับแอกกลูตินินจากเมล็ดถั่วเหลือง (SBA : soybean agglutinin) (ประมาณ 29,000 ดัลตัน) หลังจากทำ Isoelectric focusing (IEF) พบว่า pI ของ SSA มีค่าประมาณ 7.0 ส่วน SBA แยกได้ 2 แถบโปรตีนที่มีค่า pI 6.8 และ 6.65 SSA ที่แยกได้และ SBA มีสมบัติทางชีวภาพคล้ายคลึงกันมากยกเว้นค่า pH ที่เหมาะสมค่า pI และความจำเพาะต่อน้ำตาลแลคโตส นอกจากนี้ SSA ที่สกัดได้ยังมีผลยับยั้งการเจริญของสปอร์เชื้อราศัตรูพืชของถั่วเหลืองบางตัวอีกด้วย

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Surface agglutinin was extracted from soybean (Glycine max L.) seedling surface with distilled water by a 91-minute immersion. The agglutinin in the seedling was age-dependent and detected maximally in cotyledon of 3-day-old seedlings. The agglutinin can agglutinate human group A red cells and trypsin-treated rabbit erythrocytes but cannot agglutinate human group B and O red blood cells. The hemagglutinating activity was specifically inhibited by galactose and N-acetyl-D-galactosamine. The soybean surface agglutinin (SSA) was purified by ammonium sulfate precipitation and N-acetylgalactosamine affinity chromatography. The optimum temperature for hemagglutination activity by SSA was 30-40°C and the optimum pH was 8.0 Gel filtration and SDS-polyacrylamide gel electrophoresis showed that SSA contained approximately 28,000 Dalton polypeptide subunit that was quite similar to that of commercial soybean agglutinin (SBA) (MW=29,000 Dalton). Isoelectric focusing gel electrophoresis revealed the pI of SSA to be 7.0, but in the SBA, two bands of proteins which pI = 6.8 and 6.65 were found. Although the biological properties of both purified SSA and commercial SBA were quite similar, they display slight difference in pH optima, pI and lactose specificity. The purified SSA also had fungistatic effect on some soybean pathogenicfungi.

ภาควิชา ชีวเคมี ลายมือชื่อนิสิต อัญชลี เชียงเฉลา

สาขาวิชา ชีวเคมี ลายมือชื่ออาจารย์ที่ปรึกษา

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ABBREVIATIONS

SSA	=	Soybean surface agglutinin
SBA	=	Soybean agglutinin
Con A	=	Conconavalin A
PHA	=	Phytohemagglutinin
PNA	=	Peanut agglutinin
WGA	=	Wheat germ agglutinin
RCN	=	Recinus agglutinin
EPSA	=	Epicuticular plant surface agglutinin
s	=	second
min	=	minute
hr	=	hour
MW	=	Molecular weight
M	=	Molar
mM	=	Millimolar
mg	=	Milligram
ml	=	Millilitre
µl	=	Microlitre
cm	=	Centimetre
mm	=	Millimetre
U	=	unit
K_{av}	=	Relative mobility
K_A	=	Affinity constant
R_f	=	Relative mobility
i.e.	=	id est = that is
et al.	=	et ally = and coworker
e.g.	=	exempli gratia = for example
SDS	=	Sodium dodecyl sulfate
PAGE	=	Polyacrylamide gel electrophoresis
IEF	=	Isoclectric focusing
spp.	=	species
HA	=	Hemagglutination activity

V_o	=	Void volume
V_t	=	Bed volume
PDA	=	Potato dextrose agar.
Da	=	Dalton
Gal	=	Galactose
GalNAc	=	N-acetyl-D-galactosamine
PBS	=	Phosphate buffer saline
ed.	=	Editor
pp.	=	Page

CHAPTER I

INTRODUCTION

1.1 Histrorical Views of Lectins

Lectins from plants have been known for nearly a century. They were first discovered in Ricinus communis plant extract by their ability to agglutinate red blood cells and were therefore called phytohemagglutinins. There have since been many significant historical landmarks in the studies of lectins and a few are shown in Table 1. Since agglutination could, in most instance , be inhibited by simple sugars, it becomes clear that sugar residues on cell surface were the targets of lectins. Thus, they played a role in elucidating the structure of the blood group substances (Watkins,1966).

Some lectins display a considerable blood group selectivity in the human ABO system. This prompted Boyd and Shapligh (1954) to propose the term lectin which is derived from the latin word legere which means " to choose" or "to pick out". Though this term is widely accepted, it has to be kept in mind that not all lectins can differentiate ABO or other blood groups. Although most lectins have been found to specifically recognize simple sugar residues on cell surface the term lectin has now broadened to cover polyvalent carbohydrate-binding proteins or glycoproteins of non-immune origin (Goldstein et al.,1980).

Lectins were not only found in plants but also

Table 1 **Lectins: An Historical Overviews** (Zatta and Cumming, 1992)

Year	Investigator(s)	Discover
1888	H.Stillmark	Ricinus communis plant extract has hemagglutinating properties
1890	P.Erlich	Lectins as antigens in immunology
1908	K.Lansteiner and H.Raubisheck	Different hemagglutination properties in various seed extracts
1919	J.B.Sumner	Crystalization of Conconavalin A
1936	J.B.Sumner S.F.Howell	Lectins demonstrated to bind sugar Conconavalin A precipitates glycogen from solution
1940	W.C.Boyd,R.M.Reguera and K.O.Renkonen	Specificity of some lectins for some human group antigens
1954	W.C.Boyd and E. Shyleigh	The name lectin proposed instead of hemagglutinin
1960	P.C.Nowell	Lectin from Phasiolus vulgaris found to be mitogenic to resting lymphocytes
1960	J.C.Aub	Lectins preferentially agglutinate malignant cells
1976	Y.Reisner	Peanut agglutinin discriminates cortical from medullary cells in mice
1974	G.Ashwell and A.G.Morell	First mammalian lectin identified: hepatocyte asialoglycoprotein receptor specific for terminal galactose in serum glycoproteins

in living organisms. Thus, proteins from different sources and probably of different functions have been classified into one obviously artificial group. It is expected that there are at least as many different biological roles as the varieties of lectins. Moreover, some lectins may have more than one function during the life cycle of the organism (Rudiger,1984).

In animals, several lectins have been detected, isolated and characterized as molecular entities underlying biologically meaningful phenomena. So the role of animal lectins appears to be better understood than that of plant lectins. Some plant lectins are abundant in the seeds, where they may amount up to 3 % of the total weight and are easily detected by their ability to agglutinate red blood cells. Many of them are chemically well characterized but their biological function is unknown and only an object of speculation. The most prominent lectins are listed in Table 2.

1.2 Carbohydrate-Binding Properties

Earlier studies of inhibition of agglutination suggested that lectins bound to monosaccharide. However, several investigators demonstrated that the binding constant of a lectin for a specific free monosaccharide may be orders of magnitude lower than for a glycoconjugate containing that monosaccharide residues. Many other studies indicated that the binding sites of lectins are large and accommodate structurally complex carbohydrate determinants.

Table 2 Some Well-known Lectins (Rudiger,1984)

Abbreviation	Name	Plant	Binding sugar(s)

(Kornfeld and Kornfeld,1979).The monosaccharide constituents are now considered to be only part of the overall determinant.

Watkins and Morgan (1952) demonstrated that monosaccharides constitute the determinant of blood group specificity. Lectins are now used routinely in blood banks to facilitate blood typing and they can be used for the identification of blood group substances secreted in saliva and other biological fluids (Table 3).

Despite a wide diversity in three dimensional structures and in the probable binding sites among lectins, there are some features of the interaction of protein with carbohydrate that commonly appears to all lectins. Both hydrogen bonds and van der Waals interactions are involved in stabilizing these interactions (Quiocho,1986). Lectins often display stronger hydrophobic interactions with derivatives of glycosides than with the glycoside alone. For example, N-dansyl galactosamine binds 60 times stronger than N-acetyl galactosamine to Erythrina crystagalli lectin (Iglesias et al., 1982). Lectins which show similar specificity for monosaccharide can differ in their recognition of the fine structure of oligosaccharides and glycoproteins.

The majority of lectins studied so far contain metal ions, normally Ca^{2+} and Mn^{2+}(Table 4). They often require metal ions for maintenance of conformation and their ability to bind carbohydrates. Most metal ions are strongly bound and not easily removed from their site in the lectin even after long treatment with chelating agents such as EDTA.

Table 3 **Recognition Site for Lectins on Human Red Cells**

Blood type	Oligosaccharide structure
A	Fuc ↓ α1,2 GalNAc α1,3 Gal β 1,3(or4)GlcNAc β-R
B	Fuc ↓ α1,2 Galα1,3Galβ1,3(or4)GalNAc β-R
H(O)	Fuc ↓ α1,2 Gal β 1,3(or4) GlcNAc β -R

Table 4 **Metal Content of Lectins** (Adapted from Goldstein and Hayes, 1987)

Lectin from	Metal ions(atom/mol) Mn^{2+}	Ca^{2+}	Zn^{2+}
Canavalia ensiformis	4	4	_
Dolichos biflorus	0.7	3.5	0.5
Ulex enropaeus (UEA-I)	0.42	2.0	_
Griffonia simplicifolia(GS-I)	1.2	2.0	_
Erythrina cristagalli	1.0	1.9	_
Euonymus europeouos	_	8.0	0.7

The mechanism(s) by which lectins interact with monosaccharides is somewhat similar to enzyme-substrate interactions with low association constant in the order of $10^4\ M^{-1}\ s^{-1}$, two order of magnitude less than the K_A of many enzymes (Neurohr et al.,1981). The affinity constant of many lectins for an appropriate monosaccharide is approximately $10^3\ M^{-1}$ (Surolia et al.,1975). It has been shown, however, that lectins with low affinity for monosaccharide can bind with much higher affinity (K_A greater than $10^6\ M^{-1}$) to an oligosaccharide containing two or more specific monosaccharide residues with an appropriate linkage. Because lectins are multivalent, they can bind simutaneously to more than one oligosaccharide, expressing an "avidity" which can result in an increase affinity .

Surolia et al.(1975) reported that the K_A of RCA-1, a major lectin in seeds from Ricinus communis, for its binding to glycolipids in membrane is three orders of magnitude higher than that seen for lectin-binding to monosaccharide inhibitors. The binding of lectins is sensitive to host matrix composition and the nature of the receptor and in general the lectin binding is a direct function of number of receptors in membrane.

1.3 Lectin Receptors

Cell binding and agglutination by lectin is not restricted to red blood cells. Other cells of eukaryotic or prokaryotic origin are also affected (Rudiger,1984). A host

of lectin receptors has been isolated from animal or human cell membranes. Many attempts have been made to correlate their appearance to the health of the donor. Certain interaction of lectin in animal or human cell membrane may be of physiological significance. For many years it has been known that some Leguminosae seeds, for example Phaseolus valgaris , contain some toxic substance (Jaffe,1980). Janzen et al.(1976) showed that insect larvae die from adding the Phaseolus lectin to their normal diet. The same hold true for other animals and man. Phaseolus valgaris lectins react with surface of the intestinal mucosa causing damage and allow bacteria to enter (Liener,1981).

Ofcourse, protection from predators by toxic lectins only applies to a few plant species. Possibly, nontoxic lectins that evolved from a common ancestral gene and are similar in structure to the toxic ones may have other functions (Janzen, 1981).Such proposal should not be prematurely rejected since in most instance we do not know the environmental conditions under which the wild form of a lectin-bearing plant lived, let alone its evolution.

Astonishingly, the action of lectins on plant cells has been scarcely studied. Gensera et al. (1979) and Gebauer et al. (1979) can isolate proteins which are lectin binders or lectin receptors by chromatography of seed or plant extracts of several Leguminosae seeds on immobilized lectins of the same plant. These proteins are clearly different from lectins in their molecular weights as well as in their amino acid compositions and in their

actions on human or animal cells. In contrast to lectins, they do not agglutinate red cells; but like them, they affect lymphocytes, inducing enhanced mitosis. Interestingly, some are able to stimulate B lymphocytes in the absence of T cells (Gebauer et al., 1982). Though they are present in the same cell compartment as lectins, their distribution during plant development is quite different. They are present through out the life cycle of a plant in all organs in comparable concentrations (Schurz,1982). Not many receptors could be found in castor bean (Schurz,1982)or wheat (Miller and Bowles,1982). In a similar way, Bowles and Marcus (1981) isolated lectin receptors from jack beans and soybeans. Plant seed extracts prepared with denaturing buffers,however, reveal complex patterns of lectin reactive proteins (Basha and Roberts, 1981).

1.4 Detection and Assays of Lectin

The presence of lectin in a plant is readily detected by testing whether an extract of the plant agglutinates erythrocytes or some other specialized cells, and by demonstrating that the agglutination is sugar-specific, i.e.,inhibited by specific simple or complex saccharides. Hemagglutination is generally tested with native or modified erythrocytes from human and other animals, some examples are shown in Table 5.

Table 5 Agglutination Titers of Erythrocytes from Various Animal Species by Plant Extract (*)

Source of erythrocytes	Extract From			
	Bean	Pea	Lentil	Vetch
Human	800	40	20	20
Horse	16,000	128	64	128
Rabbit	8,000	1,000	2,000	200
Sheep	1,600	4	-	-
Pigeon	32,000	Weak	Weak	400
Carp	800	400	200	10
Frog	400	80	-	8

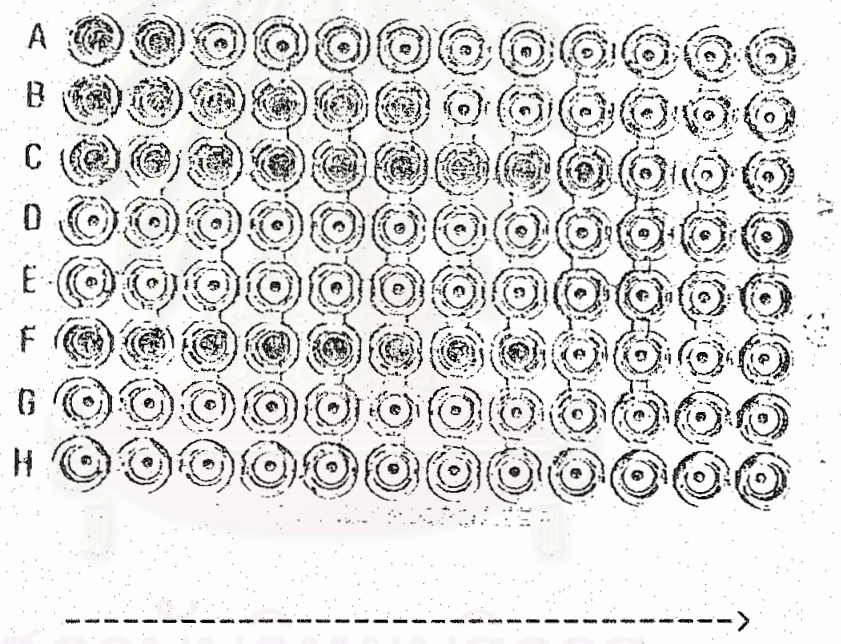
(*) Reviewed by Lis and Sharon (1979) ; agglutination titers are the highest dilution of a 1:5 saline extract causing microscopically detectable agglutination.

Blood group-specific lectins are identified with the aid of a panel of typed human erythrocytes. The most common cell modification is mild digestion with trypsin or other proteolytic enzymes (Lis and Sharon, 1972) or with neuraminidase (Marikovsky et al.,1976), an enzyme that removes sialic acid from complex carbohydrates. Such treatments leave cells intact but render them more sensitive to agglutination. The cells may also be cross-linked by glutaraldehyde or formaldehyde to stabilize them and provide a standard cell preparation that can be used for long periods of time (Liener,1975).

The hemagglutinating activity of lectins is usually measured by serial dilution technique, and the end point is determined either visually or photometrically (Figure 1) (Sharon and Lis, 1975). The visual determination is accurate only within a factor of 2, while the photometric method is more accurate but laborious and requires large amount of material. Agglutination may also be monitored automatically, using a fragiligraph (Marikovsky et al., 1976) or aggregometer (Maca and Hoak,1974).

Lectins may also be detected by their ability to form precipitates with polysaccharides or glycoproteins, either in liquid (capillary tubes) or semisolid (agar gel) media (Goldstein, 1972). Such interactions also provide information regarding lectin specificity, as well as on the constituent sugars and glucosidic linkages of the polysaccharide or glycoprotein precipitated. Here, too, the possibility of nonspecific reactions must be

Figure 1 Visual Assays on Microtiter Plates of the Hemagglutinating Activity of Soybean Agglutinin (A-D) and Peanut Agglutinin (E-H)



considered and inhibition tests with appropriate sugars carried out.

Another procedure for detection of lectins termed affinity electrophoresis, combines the principles of affinity chromatography and electrophoresis (Horejsi and Kocourek, 1974 b, Horejsi et al., 1979). In this technique, proteins are subjected to electrophoresis on a matrix formed by copolymerization of alkenyl glycosides with acrylamide. Proteins having combining sites complementary to the ligand are retarded, whereas others are not.

Qualitative immunodiffusion rather than classical hemagglutination techniques was first used by Howard et al.,(1972) to detect lentil lectin in different organs of the plant. Subsequently, radial immunodiffusion (RID) has been widely used to detect and quantify lectins in various plant extracts (Rouge and Pere ,1982). However,more sensitive radioimmunoassays (RIA) have been developed to detect Dolichos biflorus lectin (Talbot,1978) and soybean lectin from Glycine max (Pueppke et al. ,1978,Gibson et al,1982).Although specific and very sensitive,RIA technique employs sophisticated materials and could be replaced by equally sensitive enzyme immunoassays (Borrebaeck et al.,1983).

1.5 Isolation and Purification of Lectins

Isolation of lectins generally begins with the extraction of the lectin source with saline or buffer.

Preextraction with organic solvents (e.g., methanol or petroleum ether) is often used to remove lipids and other interfering substances. In the past, the isolation of lectins was achieved by conventional protein purification, such as ammonium sulfate precipitation, ion-exchange chromatography or gel filtration. At present, most lectin purification schemes employ affinity chromatography based on the ability of lectins to bind saccharides specifically and reversibly (Kristiansen, 1974). Knowledge of the sugar specificity of a lectin, which can be obtained from agglutination inhibition experiments using simple sugars and crude lectin preparations, permits the design of a suitable purification procedure. Whenever possible, commercially available adsorbents are employed. In other cases mono- or oligosaccharides, or glycoproteins such as hoggastig mucin or desialylated fetuin coupled to Sepharose, are used. Sometimes synthetic ligands are employed, e.g., N-∈- aminocaproyl derivatives of galactosylamine was used for the purification of soybean agglutinin and peanut agglutinin or N-acetylglucosamine for the purification of wheat germ agglutinin. Since the adsorbents used for the isolation of lectins may also bind glycosidases, which are frequently present in crude plant extracts, lectins purified by affinity chromatography may be contaminated by such enzymes.

1.6 Variability of Lectins

Legume lectins may seem functionally homogeneous by

virtue of binding to a particular monosaccharide, thus allowing their purification by affinity chromatography. However, electrophoretic analysis of the purified lectins may reveal extensive variations. As many as eight "isolectins" have been identified by isoelectric focusing of soybean agglutinin. Isolectin was a group of closely related proteins which had very similar composition and hemagglutinating specificity and were immunochemically indistinguishable but they differ in their isoelectric points (Goldstein and Poretz, 1986). The origin of these variations is complex and several explanations have been suggested. These include (1) genetic polymorphism, (2) post synthetic modifications, including cleavages or glycosylation, and (3) species polymorphism.

1.6.1 Genetic Polymorphism

The genome of the plant may contain several genes, each encoding a lectin or at least a protein that cross-reacts immonologically with antibodies raised against a lectin purified from the same plant. Several samples have now been described. In Phaseolus vulgaris, two different peptide chains, PHA-E and PHA-L, differing from each other by six residues at their amino terminal sequence , associate to yield five different tetrameric isolectins all present in the seeds. Likewise, from Vicia cracca seeds, Baumann et al.(1979) were able to affinity-purify two lectins, one specific for mannose and glucose and the other spacific for N-acetylgalactosamine. Electrophoretic

and sequence studies revealed a number of differences between the two proteins.The mannose/glucose-binding protein was composed of two chains, while the N-acetyl galactosamine - binding protein is a single polypeptide. The N-terminal sequences of the two lectins are homologous, but differ by 11 substitutions out of the 25 positions examined Vodkin et al (1983) and Goldberg et al (1983) have recently cloned a cDNA specific for the soybean agglutinin coding sequence. Using this cDNA as a probe, they have identified two genes, Le1, which encodes the seed lectin, and Le2, homologous to Le1 but of unknown function. It is likely that the expression of the various genes is developmentally regulated: one gene may encode a lectin expressed in seeds and another may code for protein active in roots or leaves.

1.6.2 Postsynthetic Modification

Several groups have described seed lectins composed of subunits each containing a 15,000- to 17,000-Da "long" β-chain and a 5000- to 7000-Da "short" α-chain. To this category belong the pea, the lentil, and the fava bean lectins. Antibodies raised against these two-chain proteins immunoprecipitate a single precursor polypeptide chain synthesized in an in vitro system containing mRNA from the seeds of the aforementioned plants (Strosberg,1986). Partial sequence studies using radiolabeled amino acid residues and HPLC peptide mapping suggest extensive if not complete homology between the proteins. The precursor chain is

cleaved into the α- and β-chains, which remain strongly bound to each other by noncovalent interactions.

Other types of alterations may occur after synthesis: deamination of asparagine or glutamine side chains and partial or complete glycosylation. These modifications have been mentioned to explain the frequent apperance of multiple bands in protein isoelectric focusing, a phenomenon also observed in seed lectins.

1.6.3 Species Polymorphism

Legume plant genetics was pioneered by Mendel through his famous breeding experiments on peas. Since then, efforts to obtain rigorously homogeneous species have not been a major concern. The study of the genome of plants therefore constitutes a difficult task. When several homologous lectin genes are revealed by cross-hybridization studies, it still remains to be established whether these genes are present on the same chromosome. This can only be done by *in situ* hybridization to metaphase chromosome or chromosome "walking" an approach still requiring a major undertaking.

Isoelectric focusing has already revealed striking differences between the patterns obtained with lectins from various cultivars. For example, the analysis of peanut agglutinin revealed the existence of up to eight isolectins distributed into three related isolectin profiles, which are designated the V, S, and V types. From the cultivar "Pinto III" devoid of common *Phaseolus*

vulgaris agglutinin, Pusztai et al. (1981) isolated a new type of seed lectin. The "Pinto III" seed lectin chain, although related to PHA-E, differ from it by 4 residues out of 23 when the amino terminal sequences are compared . A screen of soybean cultivars in the soybean germplasm collection of the U.S.Department of the Agriculture revealed several "Le^-" lines that lacked detectable seed lectin protein (Pull et al.,1978) and this trait followed a simple recessive mode of inheritance (Orf et al.,1978). Vodkin et al.(1983) and Goldberg et al.(1983) have now shown that certain "Le^-" cultivars possess an allelic form, le1, of the seed lectin gene Le1. This form differ from the Le1 by six base substitution and contains a 3.4 kb insertion element that interupts the coding region of the gene, thus preventing transcription of the 5'lectin sequence.

1.7 Roles in Nature

The ubiquitous occurrence of lectins in plants as well as in other organisms and their ability to discriminate among different saccharides in solution and on cell surface have prompted speculations on their physiological role (Table 6). Unfortunately, none of these is well founded so that the role of lectins in nature is still an open question.

Table 6 **Proposal for Roles of Lectins in Nature**
(Lis and Sharon,1981)

Antibodies against soil bacteria
Transport and storage of sugars
Control of seed germination and development
Regulation of plant cell extension
Attachment of symbiotic nitrogen fixing microorganisms to legumes
Protection against insect predators
Protection against fungal phytopathogens
Enzymes
Determinants of inter and intracellular recognition

There are increasing indications that lectins function in recognition phenomena, both intracellular and extracellular (Sharon,1979). An extension of this idea is the suggestion that lectins play a role in host parasite relationships, both in animals and in plants; and that they serve in the defense mechanisms of plants against pathogenic microorganisms, whether fungi, bacteria, or viruses. Recognition by lectins may also be the basis of association between legumes and their symbiotic nitrogen fixing bacteria. Of relevance in this context is the demonstration of lectins in the walls, as well as in membrane of plant cells, since it is at the cell wall that the first contact of microbial or pathogen with the host is established.

1.7.1. Binding of Nitrogen-Fixing Bacteria to Legumes

The association between legumes and nitrogen-fixing bacteria, such as rhizobia ,is specific (Lis and Sharon,1981) : legume species or cultivars which are nodulated by some Rhizobium isolates are not nodulated by others. For example , rhizobia that infect and nodulate soybeans cannot nodulate garden peas or white clover, and vice versa. The basis of this specificity may involve the capacity ofthe bacterial cells to be recognized and bound by some component, possibly a lectin (or lectins) in the roots of the plants. This is implicated by the finding that lectin from a particular legume binds only to the corresponding rhizobial species and not to bacteria that infect other legumes. Thus, fluorescein-labeled soybean

agglutinin binds to 22 out of 25 strains of Rhizobium japonicum which infect soybeans , but does not bind to any of the 23 strains from 5 species of rhizobia that infect only other legumes (Bohlool and Schmidt , 1974). These findings have led to the suggestion that soybean agglutinin is present on root tip where it serves in the spicific attachment of the symbiont bacteria to the plant (Fig2A). The attachment sites for the lectin are concentrated at one pole of the bacterium (Bohlool and Schmidt,1976; Calvert et al.,1978), in agreement with various observations that rhizobia attach end on to the surface of the host roots (Dazzo ,1978).

In another study with lectins from the seeds of four legumes (soybean, pea, red kidney bean,and jack bean) and lipopolysaccharides from the four corresponding symbiotic rhizobial species, the bacterial lipopolysaccharide bound only to the lectin from the legume with which the bacterium forms a symbiotic relationship; although the proportion of lipopolysaccharide bound was low, between 5-35 % (Wolpert and Albersheim, 1976).

A different mode of lectin action in plant-symbiont attachment has been proposed on the studies of the interaction between R. trifolii and white clover (Dazzo, 1978). According to this proposal , the lectin serves as a bridge between common or similar carbohydrate structures present on the surface of both the root tips and the bacteria (Fig. 2B). There is an immunological evidence that such a receptor is present on the cell surface of infective strains of R. trifolii, but

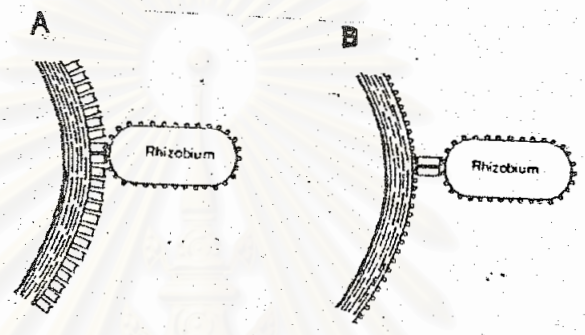


Figure 2. Mechanism of attachment of Rhizobium to plant root hairs. (A) Direct attachment of bacteria to lectin root hairs (Bohlool,1976); (B) Attachment via cross-reactive antigen present on both bacteria and root hair (Dazzo, 1978). Symbols: ⊏⊐ plant lectin : O bacterial cell surface polysaccharide or cross-reactive antigen.

absent, inaccessible, or present in reduced quantities in non-infective strains (Dazzo and Hubbell, 1975 a ; Dazzo and Brill, 1977). Further studies with the hybrid strains may help in understanding the regulation of biosynthesis and compartmentation of the carbohydrate structure present on the bacterial surface through which trifoliin attaches the cells to the plant roots.

1.7.2. Protection Against Plant Phytopathogens

It has been suggested that lectin may interfere with fungal growth and sporulation by attaching to surface of fungi covered by polysaccharides that react with the lectin . This proposal was based upon the observed inhibition of Trichoderma viridae spore germination and colony growth by wheat germ agglutinin. It has been suggested that lectins function in protecting plants against phytopathogens during seed imbibition and early seedling growth (Mirelman et al.,1975). Lectin caused interference with incorporation of exogenous radiolabeled carbon sources, and the binding of fluorescein isothiocyanate conjugated lectins to cell walls of several types of fungi (Barkai-Golan et al.,1978, Mirelman 1975). Further studies on the distribution of lectins in plants in relation to plant resistance to fungal infection, and on the effect of lectins on different fungi, are required in order to validate this hypothesis.

There have also been suggestion that lectins are involved in the defense of plants against pathogenic bacteria. Ultrastructural evidence was obtained for immobilization of the incompatable bacterium Pseudomonas pisi in tobacco leaf tissue (Goodman et al.,1976). When tobacco leaves are incubated with avirulent strains of Pseudomonas solanacearum , a pathogen of tobacco and potato plants, the bacteria rapidly attach to the plant cell walls and are then enveloped by the walls (Sequeira , 1978) . Virulent bacteria, however, remain free in the spaces between the cells , where they multiply and spread. It has therefore been postulated that avirulent strains attach to lectins in the plant. Accordingly, 55 virulent and 34 avirulent strains of P. solanacearum, from different geographic regions, representing all major races and biotypes, were tested for their interaction with potato lectin. All avirulent isolates bound to lectin and were agglutinated strongly, whereas virulent isolates were not. Binding of the lectin to avirulent cells were hapten-specific and could be inhibited by chitin oligomers. Failure of the the virulent bacteria to bind the lectin was corrected with the presence on their surface of an extracellular polysaccharide not found on the avirulent cells. Indeed, when most of the extracellular polysaccharide was removed from the virulent cells by washing, the cells were agglutinated strongly by the lectin.

Studies with the red kidney bean have shown that the saprophytic bacterium Pseudomonas putida is

immobilized and encapsulated in the intercellular spaces of the leaves of the plant, whereas the phytopathogenic bacteria Pseudomonas phaseolicola and Pseudomonas tomato do not adhere to the plant cell walls, nor encapsulated inside the cells (Sing and Schroth, 1977). It was suggested that plant lectins may be involved in the immobilization and encapsulation, since only P.putida cells were agglutinated by phytohemagglutinin.

1.8 Localization of lectin

It is generally agreed that Leguminosae lectins, the most-studied group, are preferentially localized in the cotyledons. Maximum content is reached in the mature seeds. After germination, the lectin content gradually decreases in the cotyledons, whereas the newly developing organs, leaves, stem or root contain little of any lectin (Rudiger, 1984). Thus, these proteins share the fate of storage proteins. In all these experiments, the lectins' binding specificity towards sugars or antibodies has been used. Nothing is known about the fate of the protein molecule after the binding capacity has disappeared, i.e., whether it is broken down to amino acids or only modified to related molecules that may still fulfill the same function.

Special attention has been paid to the occurence of lectins in roots. This point is of almost importance for the hypothesis that lectins play a role in establishing and/or maintaining the well known legume-Rhizobium

symbiosis. Hapner and Robbins(1979) found the root lectin from sainfoin (Onobrychis viciifolia) to be identical with the seed lectin. Gatehouse and Boulter (1980) and Kijine et al.(1980) isolated minimal amount of proteins from pea roots that are related to,but not identical with, the seed lectin. Gade et al.(1981), studying soybeans, found that the root lectin resembles the seed lectin very closely. On the other hand, Low and Strijdom (1982) described a root lectin from the legume Lotononis that is clearly different from the seed lectin. The root lectin binds to rhizobia, whereas the seed lectin does not.

Localization studies in plants other than legumes have been done with the wheat germ lectin. In the resting seed, this lectin is entirely found in the embryo. During germination and growth, it appears in a loosely bound form at the actively growing parts, preferentially at those exposed to the soil.

At present, many biological functions of plant lectins have been suggested, one of which is lectins act as primary defense mechanism of the plant against invading pathogens. In accordance with such suggested function, it was suggested that such lectin should be present on the outer surface of the cells. Thus, many studies on lectin have focused on lectins on cell surface.

The location of lectins in the cell is not yet clear. In the past, they were believed to be in the cytoplasm, but presently they are assumed to be associated with the protein bodies, the storage granules of the Leguminosae

cotyledons. Baumann and Rudiger (1981), Schurz (1982) concluded that this association is only weak and can easily be broken, leaving the protein bodies intact.

1.9 Glycine max (Soybean) Agglutinin

That extracts of soybean meal contained hemagglutinating activity was known for many years. First found associated with the toxic and growth retardation properties of uncooked soybean meal, the hemagglutination activity was subsequently shown to reside in a unique group of proteins known as soybean hemagglutinin (SBH), later renamed soybean agglutinin (SBA) (Lis et al.,1970). Isolation and purification of soybean agglutinin was achieved by many investigators, first by conventional protein purification schemes such as ion-exchange and calcium phosphate chromatography (Lis et al.,1966 ; Lis and Sharon,1973 a;) and by moving boundary electrophoresis (Wada et al.,1958). These methods have now been completely superseded by affinity chromatographic techniques.

Gordon and co-workers (1972) coupled N-(6-aminohexonoyl)-α-galactopyranosylamine to cyanogen bromide-activated Sepharose to afford a specific adsorbent for the agglutinin. A second, simpler affinity adsorbent was prepared by Allen and Neuberger (1975) by reaction of galactosamine with CH-Sepharose 4 B in the presence of carbodiimide. In both cases, elution with galactose gave a major and several minor agglutinins that were removed by

anion-exchange chromatography.

Soybean agglutinin was resolved into four hemagglutinating proteins by DEAE-cellulose ion-exchange chromatography and by isoelectric focusing (Lis et al., 1966). The isolectins are immunologically indistinguishable. Thus, it appears that soybean agglutinin exists as multiple, highly similar forms. A lectin isolated from the roots of 5-day-old soybean seedlings has properties very similar to the seed lectin (Gade et al. ,1981). Although SBA is present in seeds of most soybean lines, a few lack this lectin (Pull et al.,1978).

Soybean agglutinin is a tetrameric glycoprotein composed of equal amounts of two slightly different subunits [MW=30,000 (Lotan et al.,1974)], each of which contains an N-terminal alanine. Like several other lectins, soybean agglutinin is comparatively rich in acidic and hydroxylic acids and is devoid of cysteine (Lotan et al.,1974). In common with other legume lectins, soybean agglutinin possesses high amount of β pleated-sheet comformation(Jergensens,1978).

Soybean lectin contains 7% by weight of carbohydrate consisting of mannose and N-acetylglucosamine in a molar ratio of 9:2 (Lis,1968; Lis and Sharon, 1978). Carbohydrate - binding studies (by inhibition of hemagglutination or glycoprotein precipitation) on soybean lectin (Lis et al.,1970) showed that it exhibits greatest affinity for N-acetylgalactosamine, its glycosidase and oligosaccharides in which this sugar is present at the nonreducing terminal unit. Galactose and its

derivatives are less reactive.

Soybean agglutinin precipitated several purified blood group substances . Maximal precipitation was achieved with type A substances whereas A2 substances were considerablely less active. However, B - active substances reacted poorly, despite their content of terminal ∝-galactosyl groups.

Isolation of lectin from plants was usually performed on total homogenate of plant tissue. This method renders homogenized SBA harder to purify since there are contamination of many proteins which were not lectin. Recently, Skubatz and Kessler (1984,1988) discovered plant surface agglutinin from cucumber extracted by washing of the surface with fresh water. This agglutinin was called epicuticular plant surface agglutinin (EPSA). Lectins that may be involved in primary defense mechanism should be present on plant surface because pathogens must first attack at the surfaces before penetration to other organs.

1.10 Aim of the Thesis

As mention earlier, Soybean Agglutinin has been studied quite extensively but SBA on plant surface has not yet been reported. Therefore, this research will emphasize on the following aspects:

1. Screening of organs and ages of soybean seedlings with the highest surface agglutinin.
2. Purify and characterize the agglutinin in

comparison with other SBA reported.

3. Identify the ability of the surface agglutinin to interact with some pathogenic fungi.

CHAPTER II
MATERIALS AND METHODS

2.1 Materials

2.1.1 Biological Materials

Seeds of Soybean (Glycine max L) SJ.4 were kindly provided by Dr. Juangjan Duangpatra, Kasetsart University. The varieties of fungal spores were obtained from the Department of Plant Pathogen, Kasetsart University. Human blood was kindly provided by the Blood Bank, Thai Red Cross Organization, Bangkok. Rabbit Reticulocytes were prepared from rabbits reared in the Department of Biochemistry, Faculty of Science, Chulalongkorn University.

2.1.2 Chemicals

2.1.2.1 Chromatographic chemicals

Sephadex G-75 was the product of Pharmacia Fine Chemicals. N-acetyl-D-galactosamine cross linked 4% beaded agarose column was the product of Sigma Chemical Company.

2.1.2.2 Electrophoretic and Isoelectric focusing chemicals

Acrylamide was the products of E.

Merick Ag. N,N,N,N′-methylene bis acrylamide (BIS), Glycine, Coommasie Brillient Blue R_{250}, EDTA, β-mercaptoethanol were all purchased from Sigma Chemical Company. N,N,N′,N′-Tetramethylene diamine (TEMED) and Bromophenol Blue were from BDH Laboratory Chemical Division. Tris-HCl was the product of Fluka and Sodium dodecyl sulfate (SDS), ammonium persulfate and standard molecular weight protein markers for electrophoresis were all products of Bio-Rad.

Chemicals for isoelectric focusing gel electrophoresis were purchased from the following sources; Acrylamide from E.Merick Ag. Ampholine from LKB, Sucrose from Sigma Chemical Company, Ammonium persulfate and IEF standards were all purchased from Bio-Rad.

2.1.2.3 Carbohydrates

All sugars which were used in testing specificity of lectins, which included; rhamnose, arabinose, trehalose, mannose, xylose, fucose, galactose, mellibiose, lactose, cellobiose, glucosamine, mannosamine, galactosamine, N-acetyl-D-galactosamine and N-acetyl-D-glucosamine, were the products of Sigma Chemical Company. Soybean agglutinin (SBA) was purchased from Sigma Chemical Company.

2.1.2.4 General chemicals

Sodium phosphate, mono and dibasic were products of Merk. Sodium chloride and Sodium hydroxide

were from EKA Nobel Ltd., Ammonium Sulfate was from BDH Chemical Ltd. Phenol (Folin-Ceocalteau) was from Fisher Scientific Company. Sodium tratrate, Copper sulfate and Sodium bicarbonate were from J.T. Chemical Company. Imidazole-HCl was from Fluka.

2.2 Methods

2.2.1 Soybean Surface Agglutinin

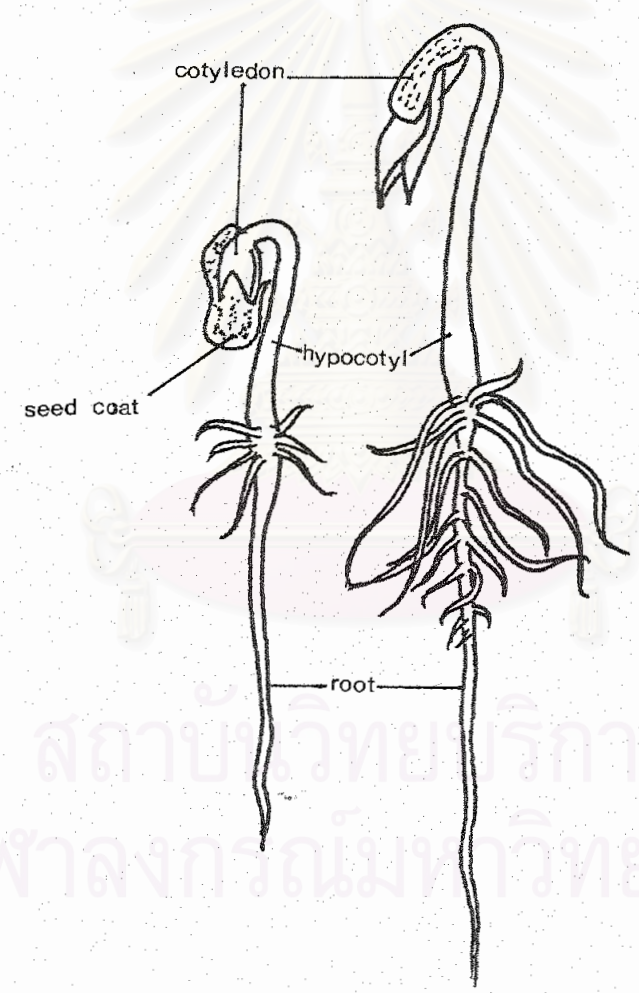
2.2.1.1 Germination of Soybean Seedling

Soybean seeds were sterilized by soaking in 90% ethyl alcohol for 5 minutes. After washing with distilled water, the seeds were soaked for 15 minutes in 3% sodium hypochlorite, washed 2-3 times with distilled water and soaked for 3-5 hrs. After washing,the seeds were germinated on two layers of moistened filter papers (Whatman No. 1) and placed on plastic boxes with transparent plastic covers for 1-2 days. Germination was carried out at room temperature (25-30 °C) in a controlled environment chamber in darkness or under 12-hr. photoperiod. Fluorescent standard cool-white lamps served as light source.

2.2.1.2 Surface Washing of Seedlings

The seedlings at appropriate ages were separated into 3 parts: cotyledon, hypocotyl and root. (Figure 3). The fresh weight of each part was recorded. Surface washing was performed on each part by immersion into distilled water with a 1:1 proportion of plant fresh weight to water. Each part of the plant was washed at four successive intervals by first immersion of the plant in distilled water for 1 minute. This first wash was called

Figure 3 Parts of Soybean Seedlings



fraction I. The plant was then transfered to a second batch of water and immersed for 30 minutes. Three successive 30 minute-washing were performed to give fraction II-IV. A total immersion of the plant with the same fresh weight in water for 91 minutes without serial transferred was also carried out (Fraction V). Each fraction was filtered and lyophylized. The dried material was dissolved in 0.02 M phosphate buffer saline (PBS) pH 7.4 with 0.14 M NaCl at a ratio of 1 ml PBS per 100 gram fresh weight to yield the crude extract of soybean surface agglutinin (SSA). After the washings,the remaining plant tissue was homoginized in 0.02 M PBS (1 gram fresh weight / 10 ml PBS) with cold pestle and mortar. The homogenate was centrifuged at 12,000g for 10 minutes and the supernatant collected called fraction VI was examined for residual HA.

2.2.2 Purification Procedure of SSA

2.2.2.1 Crude Extract

Cotyledons of soybean were harvested and extracted for surface agglutinin with 0.02 M PBS pH 7.4 at a ratio of 1 gram fresh weight per 1 ml distilled water. After 91 minutes, the solution was pooled, filtered and concentrated with a Diaflo ultrafiltration cell provided with PM-10 filters. The concentrated solution (about 10 ml) was centrifuged at 12,000 g for 10 minutes. The clear supernatant was collected and used for further study. It would be referred to as crude extract.

2.2.2.2 Ammonium Sulfate Precipitation

Solid ammonium sulfate was added to the crude extract with continuous stirring to give the desired concentration. The precipitate was collected by centifugation at 12,000 g for 20 minutes, redissolved in PBS and dialysed to remove the salt. The remainiing supernatant was futher saturated with ammonium sulfate to the desired percentage of solution. The procedure was repeated up to 80% ammonium sulfate saturation. Each precipitate suspension obtained were assayed for HA.

2.2.2.3 Affinity Chromatography

The soybean agglutinin obtained from ammnoium sulfate precipitation(2.2.2.2) was further purified by affinity chromatography at 4 °C on an N-acetyl-D-galactosamine cross linked 4 % beaded agarose column preequilibrated with PBS. After the unabsorbed proteins were removed by extensive washing with the equilibrating buffer, the lectin was eluted from the column with 0.2 M galactose in the same buffer. The pooled fractions were dialysed to remove the galactose, lyophylized and resuspended in small volume of distilled water. The purity of the preparation was checked by SDS- polyacrylamide gel electrophoresis .

2.2.3 Hemagglutination Assay

2.2.3.1 Treatments of Erythrocytes

Human blood groups A,B,O and rabbit red blood cells were centrifuged at 500 g for 5 minutes and the packed red cells were washed 3-4 times with 0.02 M PBS pH 7.4. A 2 % cell suspension of each type of blood cells were prepared with PBS .

A 2 % cell suspension of rabbit or human cells containing 1 mg/ml of trypsin was incubated at 37 ^{o}C for 1 hr. The trypsin-treated cells were washed 3 times with PBS.

2.2.3.2 Hemagglutination Assay

Hemagglutination test was performed on a microtiter plate by adding 25 μl of the sample solution to be tested in the first well of a row of sample wells on the plate. The sample was then two-fold serially diluted. Twenty five microliters of the 2 % blood cell suspension was added to each well, mixed well, and incubated for 3 hrs at room temperature. The degree of agglutination was determined by visual examination. Agglutination titer was expressed as the reciprocal of the highest dilution that yielded positive hemagglutination. Specific HA was expressed as titer per mg protein of undiluted agglutinin.

2.2.3.3 Sugar Specificity

Sugar specificity of an agglutinin is determined by the ability of the sugar to inhibit hemagglutination. The inhibition test was performed on the agglutinin extract that had a titer of 4. A volume of 25 µl of two-fold serial dilutions of each sugar solution (200 mM) were prepared on the microtiter plate. Equal volume (25 µl) of the agglutinin was added to each well. After an incubation of 1 hr at room temperature, 25 µl of 2% solution (V/V) of trysin-treated red blood cells was added to each well. The minimum amount of each sugar necessary to completely prevent hemagglutination was determined.

2.2.4 Characterization of Soybean Agglutinin

2.2.4.1 Effect of pH on Agglutinin Activity

Stability of hemagglutination activity of soybean agglutinin was examined over a wide range of pH by mixing SSA with 0.1 M of appropriate buffers which yielded the required pH's: citrate buffer (pH 3.0,5.0), phosphate buffer (pH 6.0, 7.0, 8.0), tris-HCl buffer (pH 8.0,9.0) and glycine-NaOH buffer (pH 10.0).After 1 hr of incubation at room temperature, the solutions were dialyzed overnight against 100 volumes of PBS pH 7.4 and tested for hemagglutination as discribed in 2.2.3.2.

2.2.4.2 **Effect of Temperature on Agglutination Activity**

Equal amount of SSA solutions were incubated with 0.02 M PBS pH 7.4 at temperature ranging 0-100 ^{o}C for 15 minutes before the solution were brought back to room temperature and tested for hemagglutination as discribed in 2.2.3.2

2.2.5 **Protein Determination**

Protein concentration was determined according to Lowry's method (Lowry et al.,1951) with bovine serum albumin as standard. A standard curve was prepared from BSA solution containing 10 to 100 ug protein in a final volume of 0.1 ml. Three millilitres of solution A containing 1:1:100 of 0.5% $CuSO_4.5H_2O$: 1% sodiumpotassium tartrate : 2% Na_2CO_3 anhydrous in 0.1 N NaOH was added, mixed and left standing for 10 minutes. Phenol reagent (2N, 0.3 ml) was added and mixed. After 30 minutes,the absorbance at 650 nm was measured. Appropriate amount of the sample was similarly treated and the amount of protein determined from the standard curve.

2.2.6 **SDS - Polyacrylamide Gel Electrophoresis**

Molecular weight and purity of SSA was determined by SDS-PAGE followimg the method of Laemli (1970). Biorad Mini- Protein II system was used. Slab gel

(7x6x0.05 cm)was prepared with 12% seperating gel and 3% stacking gel(7x2x0.05 cm); the compositions of which are shown in Table 7.

Table 7 Composition of Slab Gel for Biorad Mini-Protein II System

Composition	3% gel (ml)	12% gel (ml)
30% Acrylamide 0.8% bis	1.5	4.2
Tris-HCl buffer pH 6.8	3.5	-
Tris-HCl buffer pH 8.8	-	2.5
1% SDS	1.5	1.0
Distilled water	5.1	2.1
EDTA	0.1	-
10% Ammonium persulfate	0.3	0.3
TEMED	5 µl	5 µl
Total	12.0	10.0

Sample of standard proteins mixture, purified SSA and commercial SBA were mixed with sample buffer (consists of 2.5% SDS, glycine, 5% β-mercaptoethanol and bromophenol blue) at the ratio of 5 volume of protein sample to 1 volume of sample buffer and boiled for 2 minutes before loaded to the gel. The gel was run at constant current of 15 mA per gel until the dye run

to the bottom of the gel, using SDS-Tris-glycine pH 8.3 (composed of 0.025M tris, 0.192M glycine) as electrode buffer. The gels were stained in 0.05% Coomassie brilliant blue R dissolved in 45% methanol, 10% glacial acetic acid. Destaining was accomplished after several changes of destaining solution containing 30% methanol, 20% glacial acetic acid.

2.2.7 Isoelectric focusing (IEF) in Polyacrylamide Gel

IEF electrophoresis was performed on horizontal mini-IEF system (Biorad).Appropriate amount of purified SSA, commercial SBA and standard pI proteins at various concentrations were applied to polyacrylamide gel plates containing ampholines in linear pH gradient at a range of 6-8 obtained from Biorad. Samples of 10 ul were applied to 5 x 10 mm sheets. The isoelectric focusing was performed at 8 $^{\circ}$ C for 1.5 hr at 100 V after an initial run at 20 mA for 15 minutes, and finished by a 60 minutes run at 400 V. Then the gel was stained with Coomassie brilliant blue R and destained with destaining solution containing 30% methanol and 20% glacial acetic acid.

2.2.8 Gel Filtration

Gel filtration was performed on Sephadex G-75 column. Sephadex G-75 was suspended in 10 mM imidazole buffer pH 6.5 for one night, then the gel suspension was packed in a glass column (1.7 x 50 cm).Head pressure was

adjusted at 20 centimatres and flow rate was 30 ml per hr. The column was equilibrated with 10 mM imidazole buffer pH 6.5. Then, 2 ml of purified SSA or standard proteins were applied to the column and the column was washed with 10 mM imidazole buffer pH 6.5. Fraction of 2 ml were collected and monitored for protein at 280 nm. Blue dextran and $K_2Cr_2O_7$ were used to determine void volume (V_o) and bed volume (V_t), respectively. To determine the molecular weight of SSA, the K_{av} of molecular weight marker proteins were plotted on semilog scale and the molecular weight of SSA was determined from the standard curve using the calculated K_{av}.

2.2.9 Cellophane - Transfer Bioassay for Fungistatic Test

2.2.9.1 Preparation of Spot Plate

Effect of substances on fungal growth was carried out using the cellophane - transfer bioassay (Needly, 1966). The cellophane used is 23 nm thick, transparent and readily absorbent. Folded sheets of cellophane were cut into disks with diameter of 5 mm, placed in distilled water and sterilized for 5 minutes in boiling water. The water was decanted and the disks were placed on moist filter paper in a culture plate (9-cm diameter).

White porcelain spot plates, each 112 mm long and 92 mm wide with 12 depressions 5 mm deep

were used. One filter paper disk (Whatman No. 42), 12.7-mm diameter,was placed into each depression. The filter-paper disk was saturated with, but not floating in the test solution (50-25 µl). Single cellophane disk was transfered with forceps from the culture plate and placed on top of each filter paper disk in the spot plate, three cellophane disks were placed on each paper disk.

2.2.9.2 Preparation of Spore Suspension

The fungi of interest were grown in 9-cm culture plates of potato - dextrose agar (PDA) prepared from raw potatoes, at 25°C for 7-14 days, depending upon the maturation of conidia. The colonies were flooded with sterile distilled water and agitated with a rubber policeman.The spore suspension was decanted into Erlenmeyer flasks, and the spore concentration was adjusted through these standards : large spores, 20 per field at 100 x magnification ; moderately large spores, 5 per field at 400 x ; small spores, 10 per field at 400 x.

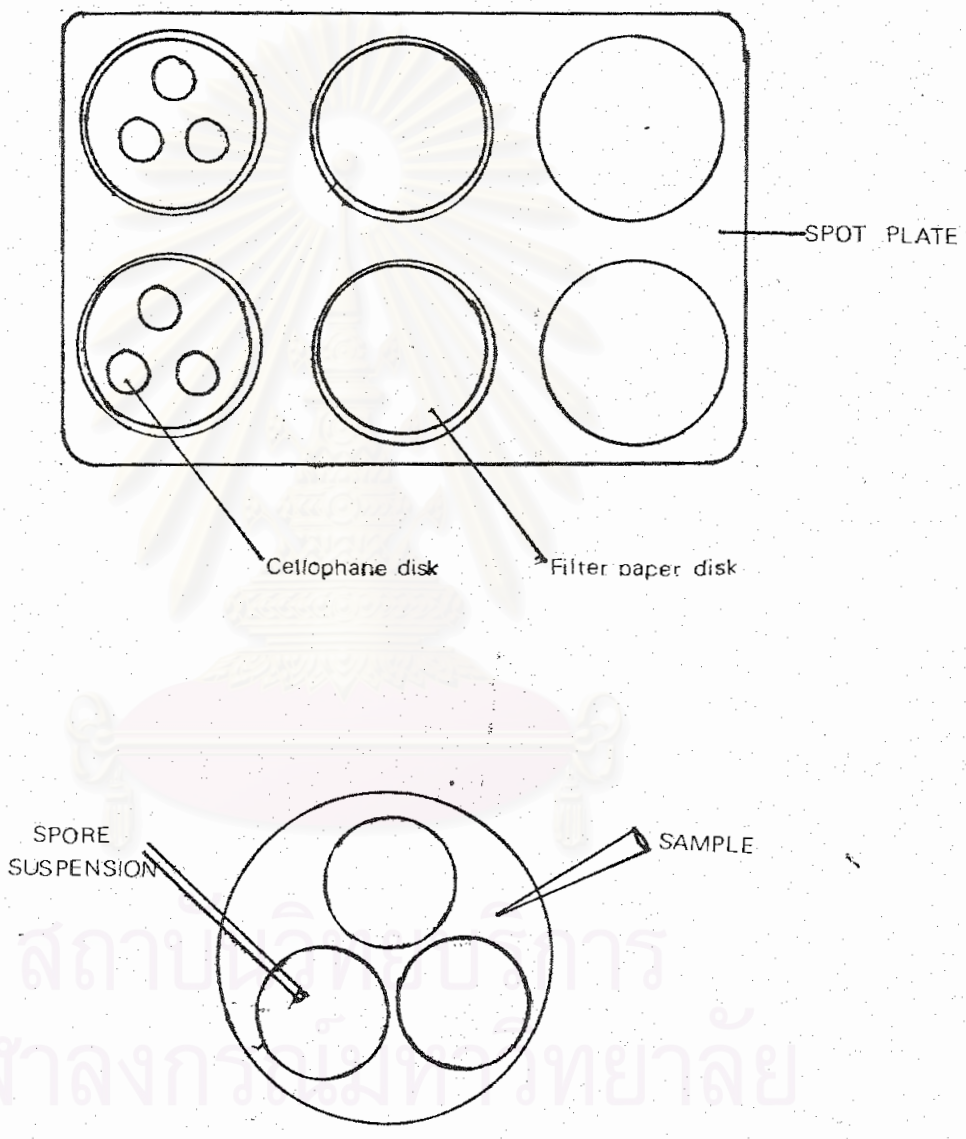
2.2.9.3 Fungistatic Test

The spore suspensions (prepared in 2.2.9.2) were transferred to the cellophane disks prepared in 2.2.9.1 with glass capillary tubing having a 0.85-mm inside diameter. Each cellophane disk was lightly touched with the end of the capillary tube, depositing approximately 0.4 µl of the spore suspension (Figure 4).

The spot plates were then stacked into a moistened chamber (glass desicators or plastic containers lined with moistened paper towel) and incubated at 25°C.

The fungistatic effect of the SSA on each fungi was determined 20 - 25 hr after the cellophane disks were seeded. The cellophane disks were transfered from the paper to microscope slides, covered with a cover slip and stained with aniline blue. The number of fungal spores on each cellophane disk were examined. If the spores did not germinate or had germ tubes that were less than half the spore length, the test sample was considered fungistatic. Degree of fungistatic effect was calculated as percentage of non-germinated spores to total spores appeared in the microscopic field.

Figure 4 Cellophane - Transfer Bioassay Technique



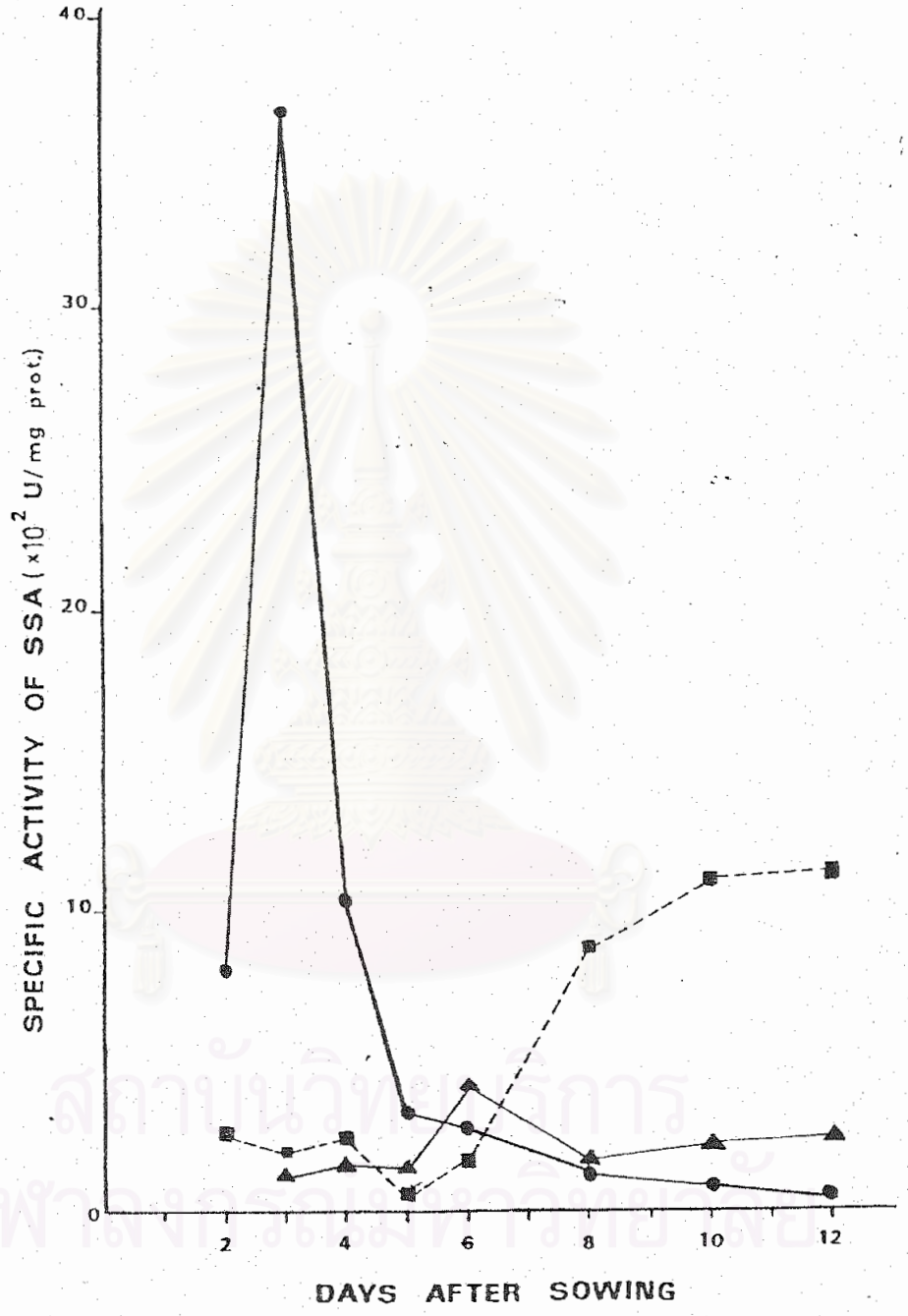
CHAPTER III

RESULTS

3.1 Surface Agglutinin in Soybean Seedlings

3.1.1 Surface Agglutinin from Different Parts of Soybean Seedlings

In order to determine the presence of agglutinins on the surface of different parts of soybean seedlings, the plantlets were separated into various portions and immersed in distilled water for 91 minutes as described in section 2.2.1.2. Agglutinin activities were detectable in surface extracts of cotyledons, hypocotyls and roots (radicles) on the second day after germination (Figure 3). Agglutinin was significantly detected in surface extract of cotyledon at the age of 2-6 days old with a maximum at 3 days old. The activity in roots was detectable at the age of 6 days onward with gradual increase; while the hemagglutinating activity in hypocotyls was very low at all ages.


40
30
20
10
0
SPECIFIC ACTIVITY OF SSA (×10² U/mg prot.)
2
4
6
8
10
12
DAYS AFTER SOWING

3.1.2 Cotyledon Surface Agglutinin at Different Times of Extraction

Cotyledons were selected for further extraction of surface agglutinin in soybean seedlings since the highest agglutination activity was shown in 3 - day old (Figure 5). Surface washing of the cotyledon for differentdurations of immersion in distilled water was performed to determine the optimum time for extraction.

Cotyledon from 3 days old seedlings was immersed in different changes of distilled water at the following intervals(Figure 6): 1-minute wash (fraction I), followed by three successive 30-minute wash (fraction II-IV) and a single wash of 91- minute (fraction V). Fraction VI was the homogenate of cotyledon after the 91 minutes wash. The result confirmed that immersion of cotyledon in distilled water for 91 minutes yielded the highest hemagglutination activity.Residual agglutinin in the tissue homogenate of cotyledon after 91 minutes wash (fraction VI) was minute. Therefore, the single 91 - minute wash of cotyledon surface of 3 - day old seedlings will be used for further study of SSA.

Figure 6 Hemagglutination Activity of Surface Agglutinin from 3- Day Old Soybean Cotyledon at Different Extractions Times.

Washing of surface agglutinin from cotyledon of 3 days old seedlings was performed as discribed in section 2.2.1.2.

I = 1 minute wash from 0 - 1 minutes

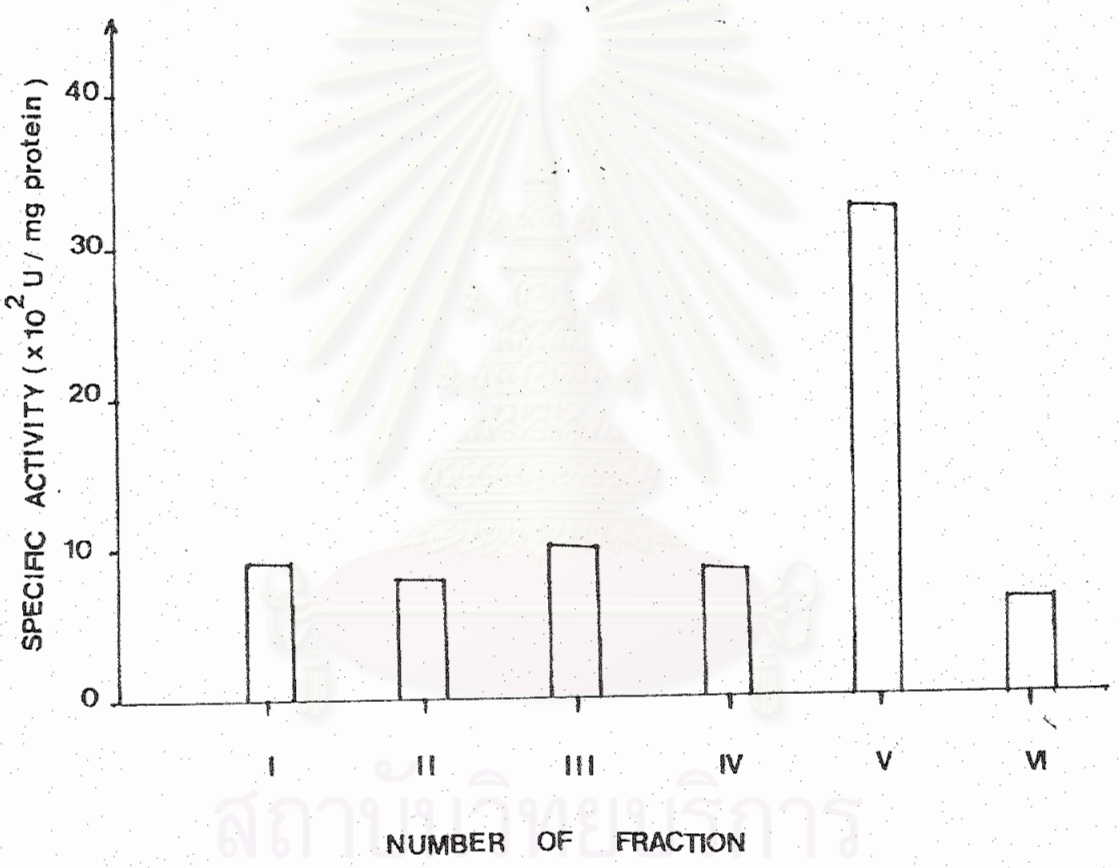
II = 30 minutes wash from 2 - 31 minutes

III = 30 minutes wash from 32 - 61 minutes

IV = 30 minutes wash from 62 - 91 minutes

V = 91 minutes wash from 0 - 91 minutes

VI = homogenate of cotyledon after subjected to the total 91 minutes wash



3.1.3 Screening of Cells for Agglutination Test

To screen for suitable cells which can be used for HA test, experiments were carried out on the ability of cotyledon surface extract to agglutinate red blood cells from different sources. Solution of 12 ug/ml of cotyledon surface extract was used in the examination of red cell specificity: Experiment was performed on microtiter plate as described in section 2.2.3.2. The cotyledon extract agglutinated only human blood group type A with no activity on human blood group B, O or untreated rabbit erythrocytes (Table 8). However, HA activity of the extract on trypsin-treated rabbit erythrocytes was especially high while enzyme treated human red cells showed no recognition. Therefore trypsinized rabbit erythrocytes were used for future HA test of the surface agglutinin.

Table 8 Hemagglutination Activity of Cotyledon Extract on Different Red Blood Cells

Source of Red blood cells	Specific HA (U/mg protein)	
	Untreated RBC	Trypsinized RBC
Human blood group A	3,200	-
B	0	-
O	0	-
Rabbit RBC	0	25,600

3.1.4 Sugar Specificity of the Agglutinin

To determine sugar specificity,various sugars were tested for ability to inhibit the hemagglutination activity of crude cotyledon surface extract using the method described in section 2.3.1.

As shown in Table 9, glucose, mannose, fructose, rhamnose, fucose, trehalose, arabinose, xylose, sucrose, cellobiose, melibiose, glucosamine, galactosamine, manosamine and N-acetyl-D-glucosamine did not inhibit agglutination at final concentration as high as 5 mM. On the other hand , galactose and N-acetyl-D-galactosamine (GalNAc) can inhibit the agglutination reaction even at a minimum final concentration of 0.08 mM and 1.25 mM respectively.

Table 9 Sugar Specificity of Cotyledon Surface Agglutinin

Sugar(*)	Inhibition on HA
Monosaccharide	
L-rhamnose	-
D+arabinose	-
L-arabinose	-
D+trehalose	-
D+mannose	-
D+xylose	-
D+fucose	-
D+galactose	+
Disaccharide	
D+mellibiose	-
D+lactose	-
D+cellobiose	-
Aminosugar	
D+glucosamine	-
D+mannosamine	-
D-galactosamine	-
N-acetyl glucosamine	-
N-acetyl galactosamine	+

* maximum concentration used = 200 mM

+ indicates inhibition of HA

- indicates no effect on HA

3.2 Purification of the Cotyledon Surface Agglutinin

The crude extract of soybean cotyledon surface agglutinin investigated and selected in section 3.1 was subjected to first step of purification by ammonium sulfate precipitation.

3.2.1 Ammonium Sulfate Precipitation

The surface washing from 3-day old cotyledon were pooled and precipitated with ammonium sulfate at various concentrations (section 2.2.2.2). The precipitation was performed by stepwise increase of ammonium sulfate: 0-30%, 30-40%, 40-50%, 50-60%, 60-70% and 70-80% saturation. The precipitates obtained were resuspended in PBS and assayed for HA after removal of salt by dialysis. Agglutination activity was maximally precipitated at the 60%ammonium sulfate as shown in figure 7. At 70-80% ammonium sulfate, agglutination activity completely deminished. As the surface extract contained minute amount of protein, it was dicided to avoid loosing too much of the lectin. Therefore, precipitation of HA in the crude extract will be collected at one step of precipitation at 50-70% ammonium sulfate.

Figure 7 **Ammonium Sulfate Precipitation of Surface Agglutinin of 3 Days old Soybean Cotyledon**

Cotyledon surface extract was precipitated at different concentrations of ammonium sulfate increased at 10% increment from 0 to 80% (see section 2.2.2.2).

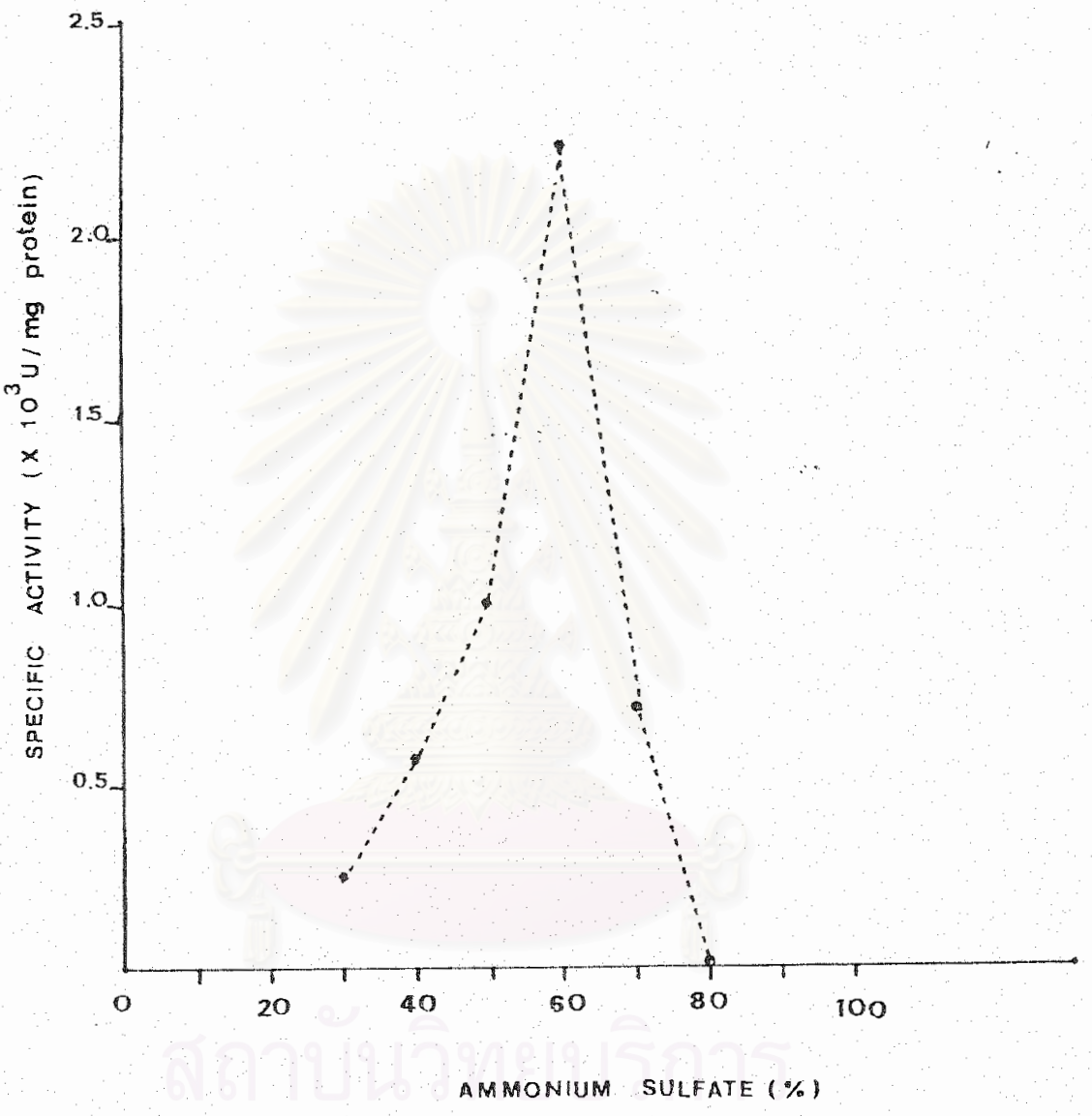


Figure 8 Chromatographic profile of Agglutinin from Soybean Cotyledon on N-acetyl-D-galactosamine Cross Linked 4% Beaded Agarose Column

Ammonium sulfate precipitate of crude cotyledon surface agglutinin from 3 days old soybean seedling was subjected to N-acetyl-D-galactosamine column and washed with phosphate buffer pH 7.4 to remove unbound protein and the bound protein eluted with galactose as described in section 2.2.2.3.

3.2.2 Affinity Column Chromatography

As the hemagglutination activity of crude surface extract of cotyledon was shown to be highly specific to N - acetyl - D - galactosamine and galactose (Table 9), the next appropriate step to apply for purification of the agglutinin was affinity column chromatrography.

The 50-70% ammonium sulfate precipitated agglutinin was subjected to affinity chromatography on an N-acetyl-D-galactosamine cross linked 4% beaded agarose column prepared in section 2.2.2.3. The column was washed with 0.02 M phosphate buffer and eluted with different concentration of galactose. Galactose was used as eluting sugar as the agglutinin also showed high specificity to the sugar and it is cheaper. The hemagglutination activity was eluted at 0.2 M galactose. The chromatographic profile is shown in Figure 8 . The agglutinin eluted from the column was called soybean surface agglutinin (SSA). Table 9 summarized all steps of purifications of SSA. The yield of SSA obtained from affinity column was 27% with 12 folds purification.

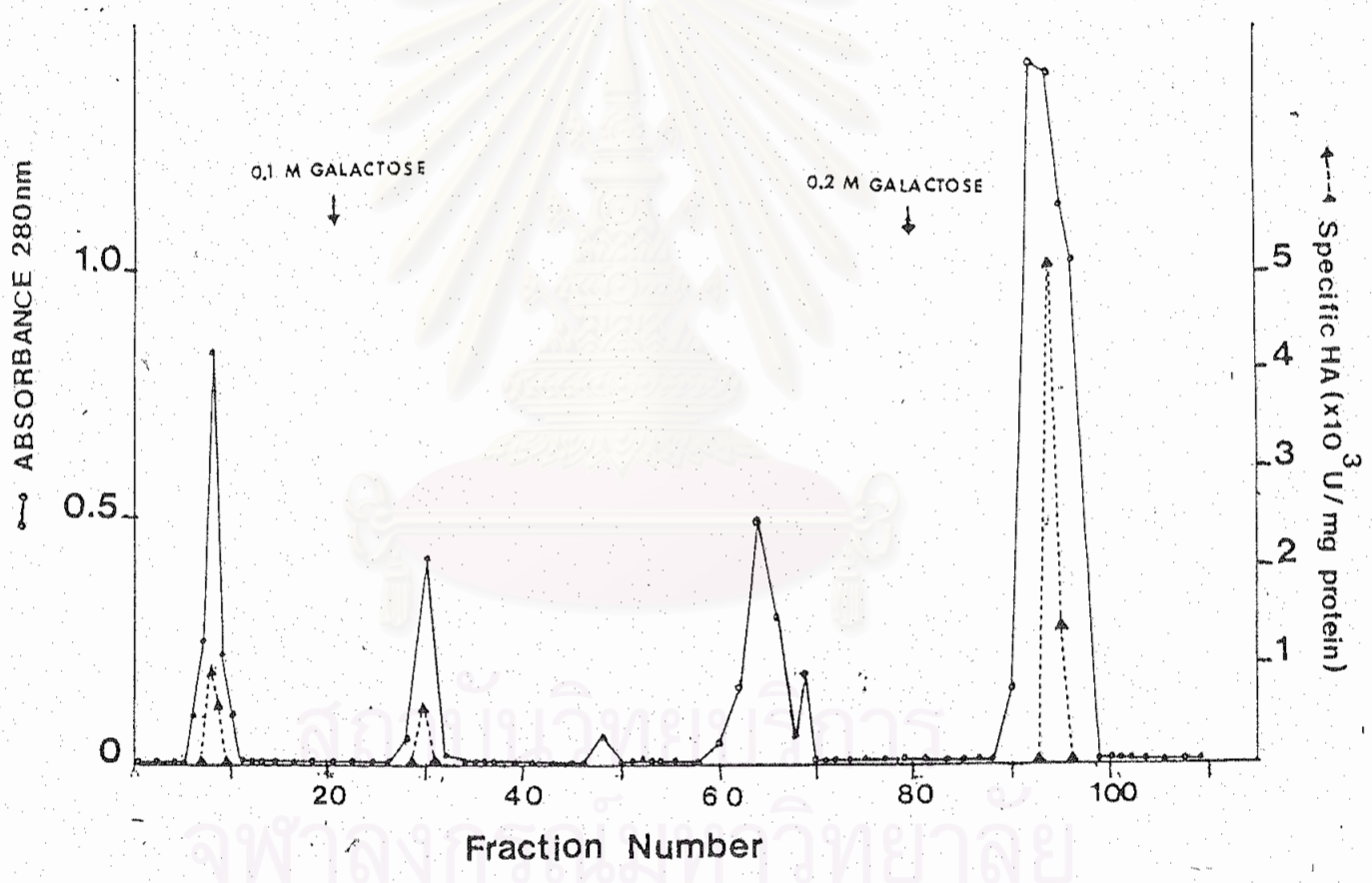

0.1 M GALACTOSE
0.2 M GALACTOSE
ABSORBANCE 280nm
1.0
0.5
0
20
40
60
80
100
Fraction Number
Specific HA (x10 3 U/mg protein)
5
4
3
2
1

Table 10 Purification of Surface Agglutinin from Soybean Seedlings.

Purification Procedure	Protein (mg/ml)	Total Protein (mg)	HA(*) (U/ml)	Total HA(U)	Yield (%)	Specific HA (U)	Fold of Purification
Crude Extract	0.63	31.50	960	48,000	100	1,523.81	1
50-70% $(NH_4)_2SO_4$ precipitation	0.58	4.06	5,120	35,840	75	8,827.59	6
Affinity column	0.52	1.04	6,400	12,800	27	12,307.69	8

(*) HA = Hemagglutination Activity

3.3 Characterization of SSA

As soybean agglutinin has been extracted and purified from seeds of Glycine max in the past, the characterization of SSA from section 3.2 will be carried out in parallel with soybean agglutinin obtained commercially which will be called SBA.

3.3.1 Thermostability of SSA

SSA and SBA were incubated at various temperatures for 15 min, cooled to room temperature and assayed for HA activity. Hemagglutination activity of SSA was found to be retained at temperatures up to 60°C. Boiling of SSA within 15 min. inactivated HA. The optimum temperature for agglutination activity of SSA was 40°C (Figure 9). Commercial SBA showed similar thermostability profile.

3.3.2 pH Stability

SSA and SBA were subjected to different pH's at 30 °C according to the procedure in 2.2.4.1 and HA was assayed. SSA was found to be quite stable over the pH range 7.0 - 9.0 with optimum pH at pH 8.0 (Fig. 8). Commercial SBA showed a slight shift of pH profile to the acidic side with maximum HA at pH 7.0. HA of commercial SBA was significantly retained in the pH range 6.0 - 9.0.

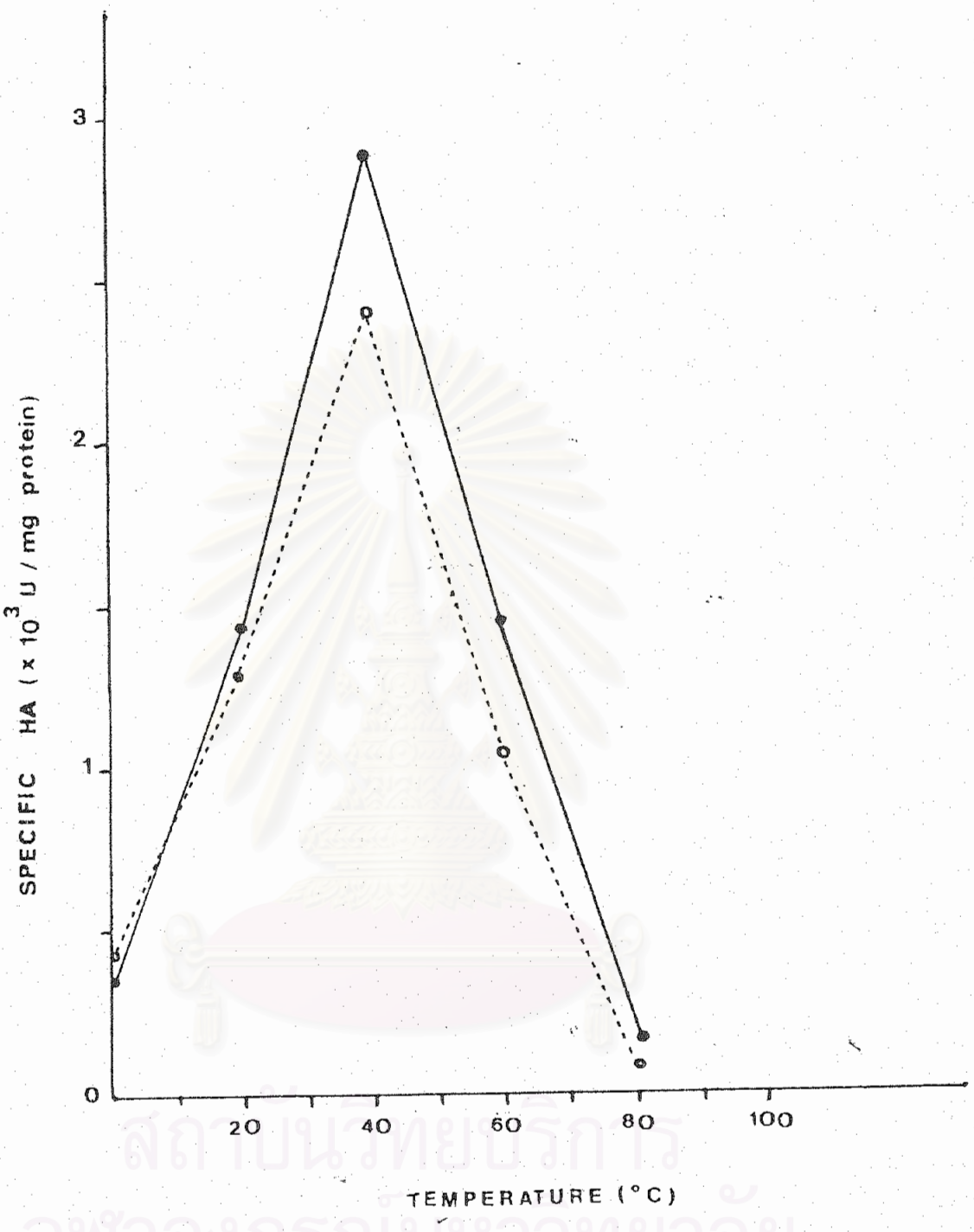


Figure 9 Effect of Temperatures on SSA and Commercial SBA Activity.

SSA and SBA were incubated at temperatures from 0 to 100°C as described in section 2.2.4.2.

●—● Commercial SBA

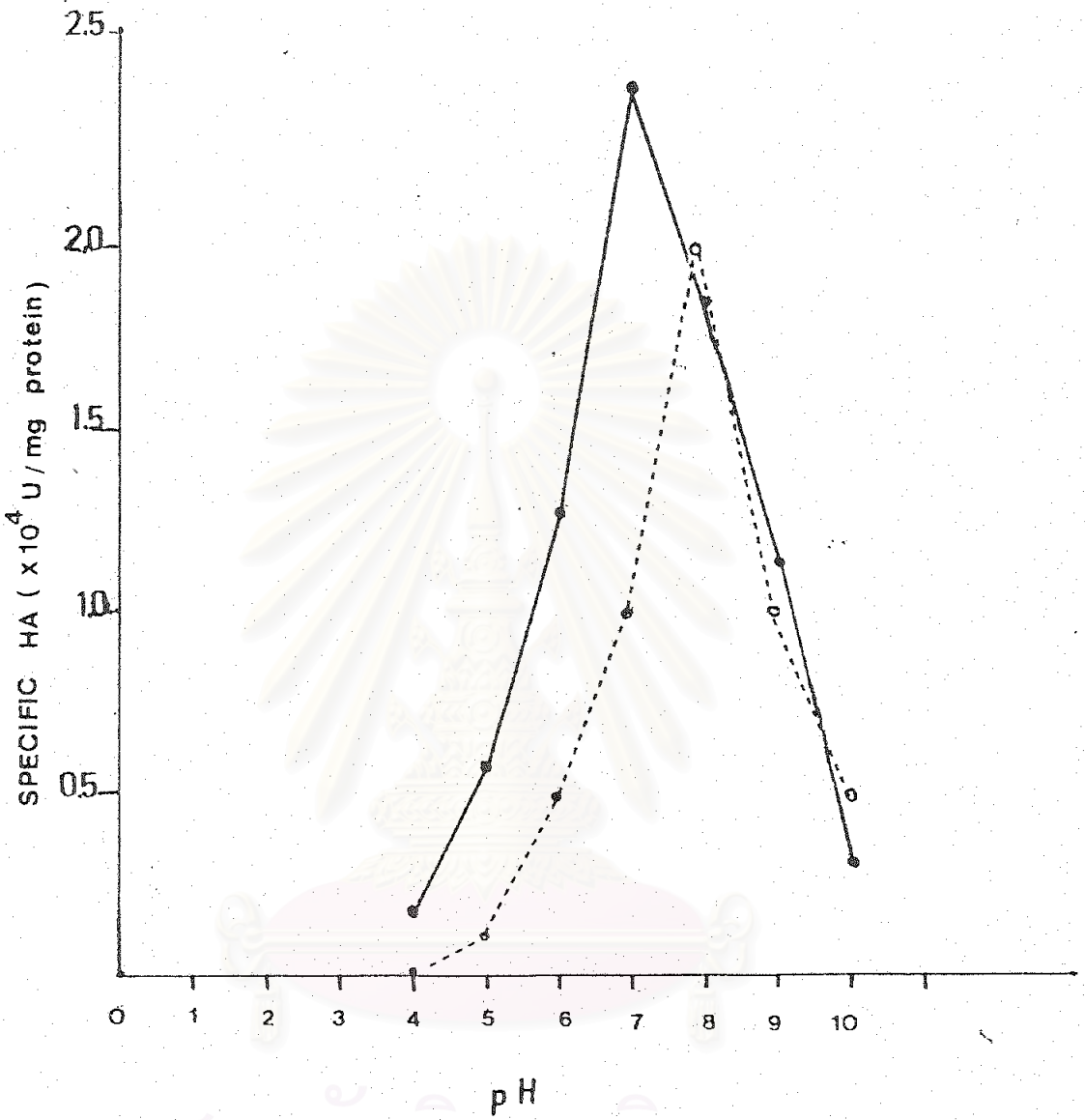
○--○ SSA

Figure 10 Effect of pH on Specific Hemagglutination Activity of SSA and Commercial SBA

SSA and SBA were subjected to different buffers at varying pH's and assayed for HA according to section 2.2.4.1.

●—● Commercial SBA

○--○ SSA



3.3.3 Molecular Weight Determination

After purification by affinity chromatography molecular weight of SSA was determined on Sephadex G-75 gel permeation chromatography.

A molecular weight calibration curve was constructed with a set of standard proteins and their K_{av} as shown in Table 11 and Figure 12. The molecular weight of affinity-purified SSA as determined from the calibration curve is 28,000 Dalton (Figure 11, 12).

Figure 11 Gel Filtration Chromatography of SSA on Sephadex G-75

Blue dextran and $K_2Cr_2O_7$ were used to determined void volume (V_o) and bed volume (V_t), respectively. One ml of fraction volume was collected from 1.7 x 50 cm glass column.

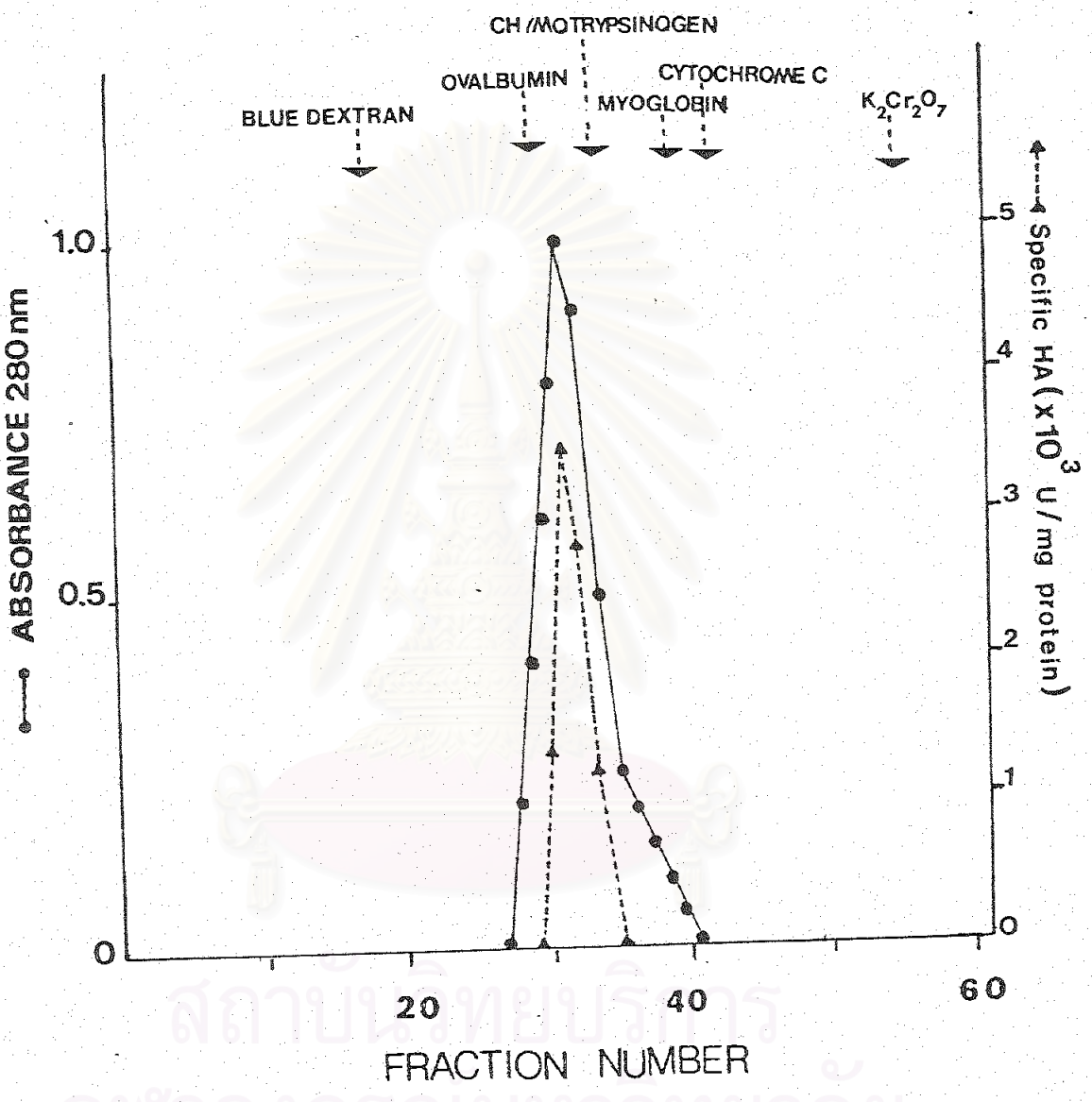


Table 11 Elution Volume and K_{av} of Protein Samples on Sephadex G-75 Gel Permeation Chromatography and Their Molecular Weight

Protein	MW(Dalton)	V_o(ml)	V_t(ml)	V_e(ml)	K_{av}(*)
Cytochrome C	12,000	46	120	86	0.54
Myoglobin	17,500	40	120	80	0.50
Chymotrypsinogen	27,000	40	120	70	0.39
Ovalbumin	43,000	46	120	60	0.19
SSA	-	38	112	64	0.34

* $K_{av} = \frac{V_e - V_o}{V_t - V_o}$

Figure 12 **Calibration Curve for Molecular Weight Determination on Sephadex G-75**

The relative mobility (K_{av}) calculated in Table 11 were plotted on a semi log scale against molecular weight of the standard proteins. The molecular weight of SSA was extrapolated from the calibration curve using its K_{av} values.

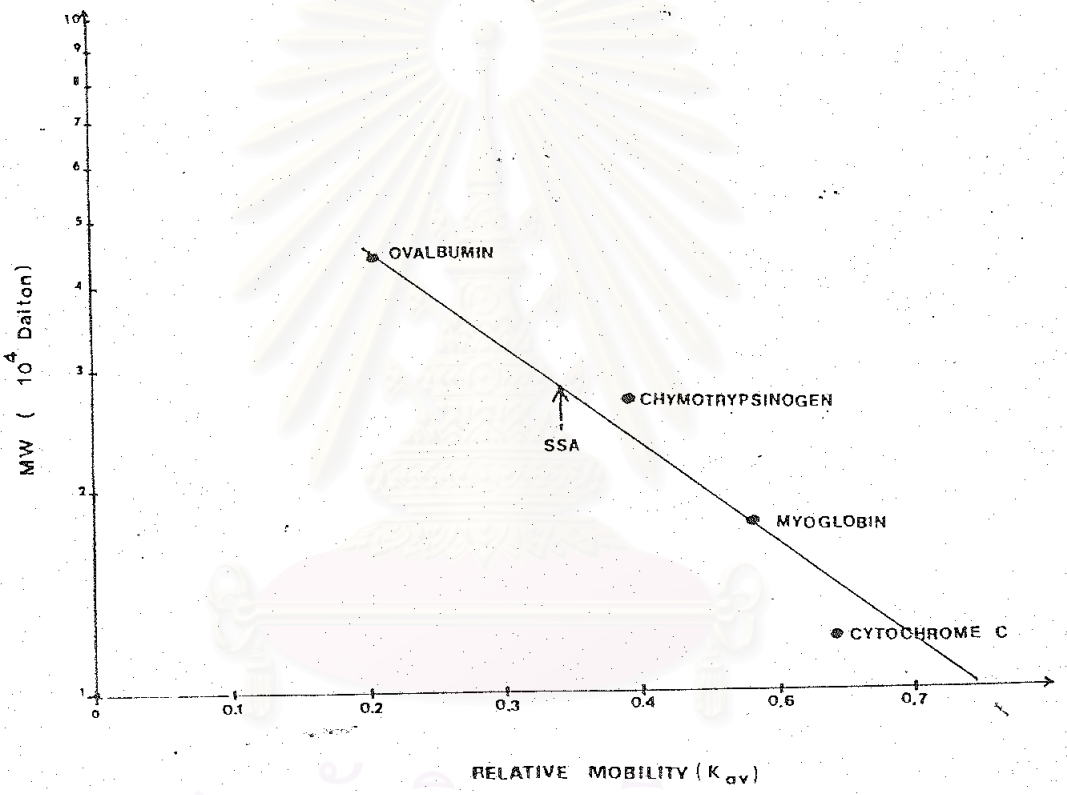


Figure 13 SDS-PAGE of Soybean Surface Agglutinin

The sample proteins were separated on 12% SDS - polyacrylamide gel electrophoresis. Twenty ul of the affinity-purified SSA, commercial SBA , crude extract and ammonium sulfate precipitated agglutinin were treated as described in section 2.2.6.

Lane A : Standard proteins mixture containing;

1) Rabbit muscle phosphorelase B (MW=97,400)
2) Bovine serum albumin (MW=66,200)
3) Hen egg white ovalbumin (MW=45,000)
4) Bovine carbonic anhydrase (MW=31,000)
5) Soybean trypsin inhibitor (MW=21,500)
6) Hen egg white lysozyme (MW=14,400)

Lane B : Crude soybean surface extract

Lane C : 50-70% ammonium sulfate precipitation agglutinin

Lane D : Affinity - purified SSA

Lane E : Commercial SBA

3.3.4 SDS-PAGE of Purified SSA

The molecular weight and purity of SSA was determined by SDS-PAGE. Sample of 40 µg proteins were subjected to electrophoresis on 12% SDS-polyacrylamide gel as described in section 2.2.6, the protein patterns were shown in Figure 11. A molecular weight calibration curve was drawn between the log of MW of standard proteins and their relative mobility (R_f) (Figure 12). The MW of SSA determined from the calibration curve was 27,500 Da while that of commercial SBA was 29,000 Da.

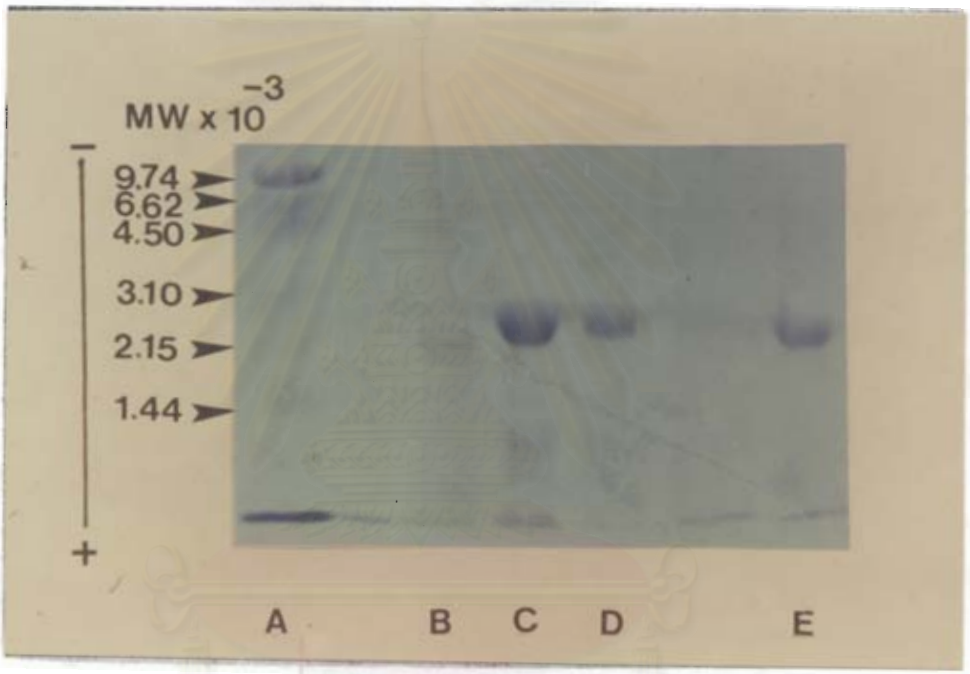
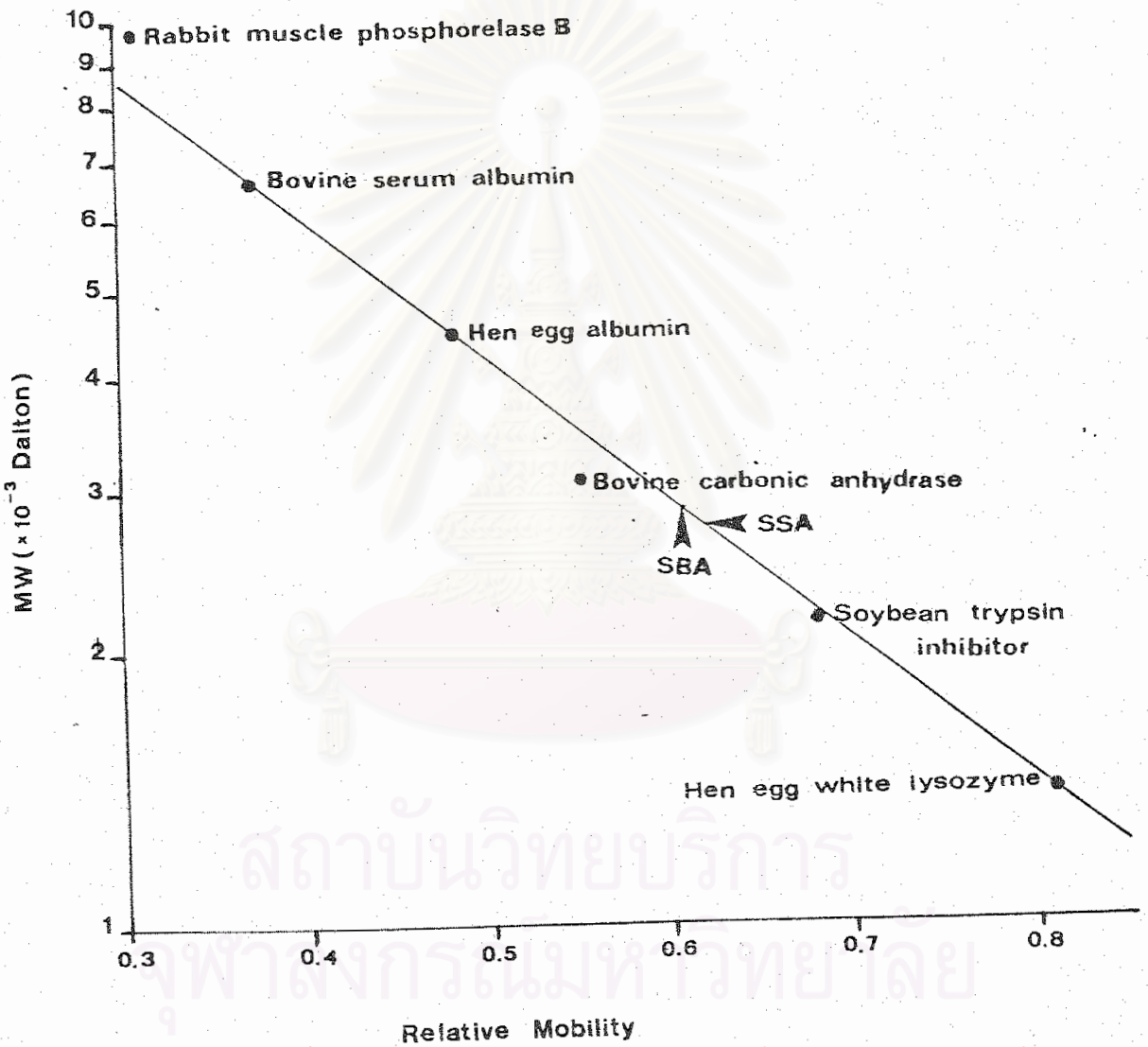


Figure 14 Calibration Curve for Molecular Weight Determination on SDS - PAGE

The relative mobility (R_f) were plotted on a semilog scale against molecular weight of the standard proteins.



3.3.5 Isoelectric Focusing (IEF) of SSA

The pI of SSA was determined by IEF gel electrophoresis. Sample of 10 µg proteins were applied to the polyacrylamide gel plates containing ampholines in linear pH gradient at a range of 6-8 as described in section 2.2.7. A pI calibration curve was drawn between the pI of standard proteins and their migration distance from the cathode. The pI of SSA and SBA was extrapolated from the calibration curve. The pI of SSA determined from this gel pattern was 7.00 . Commercial SBA showed at least 2 bands of proteins with pI's of 6.80 and 6.65.

Figure 15 **Isoelectric Focusing Patterns of Soybean Agglutinins.**

Horizontal analytical IEF was performed on polyacrylamide gel containing linear pH gradient from 6-8 according to section 2.2.7.

Lane A : IEF standard proteins content ;

1) Cytochrome C (pI=9.60)
2) Lentil lectin (pI = 8.20, 8.00, 7.80)
3) Human hemoglobin C (pI = 7.50)
4) Human hemoglobin A (pI = 7.10)
5) Equine myoglobin (pI = 7.00)
6) Equine myoglobin minor band (pI = 7.00)
7) Human carbonic anhydrase (pI = 6.50)
8) Bovine carbonic anhydrase (pI = 6.00)
9) β-Lactoglobulin B (pI=5.10)
10) Phycocyanin (pI=4.65)

Lane B : Commercial SBA

Lane C : Affinity-purified SSA

Lane D : Crude soybean surface extract

Lane E : 50-70% ammonium sulfate precipitated agglutinin

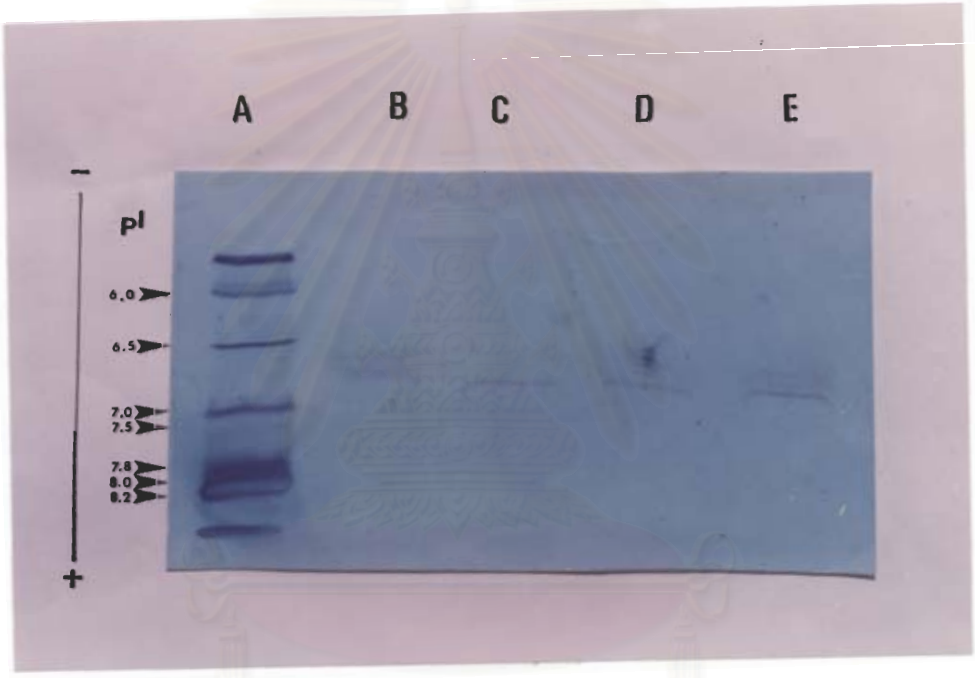


Figure 16 Calibration Curve for pI Determination on IEF-polyacrylamide Gel

The migration distance from the cathode of the standard proteins were plotted against their pI's. The pI of SSA and SBA were extrapolated from the calibration curve using their migration distance from the cathode.

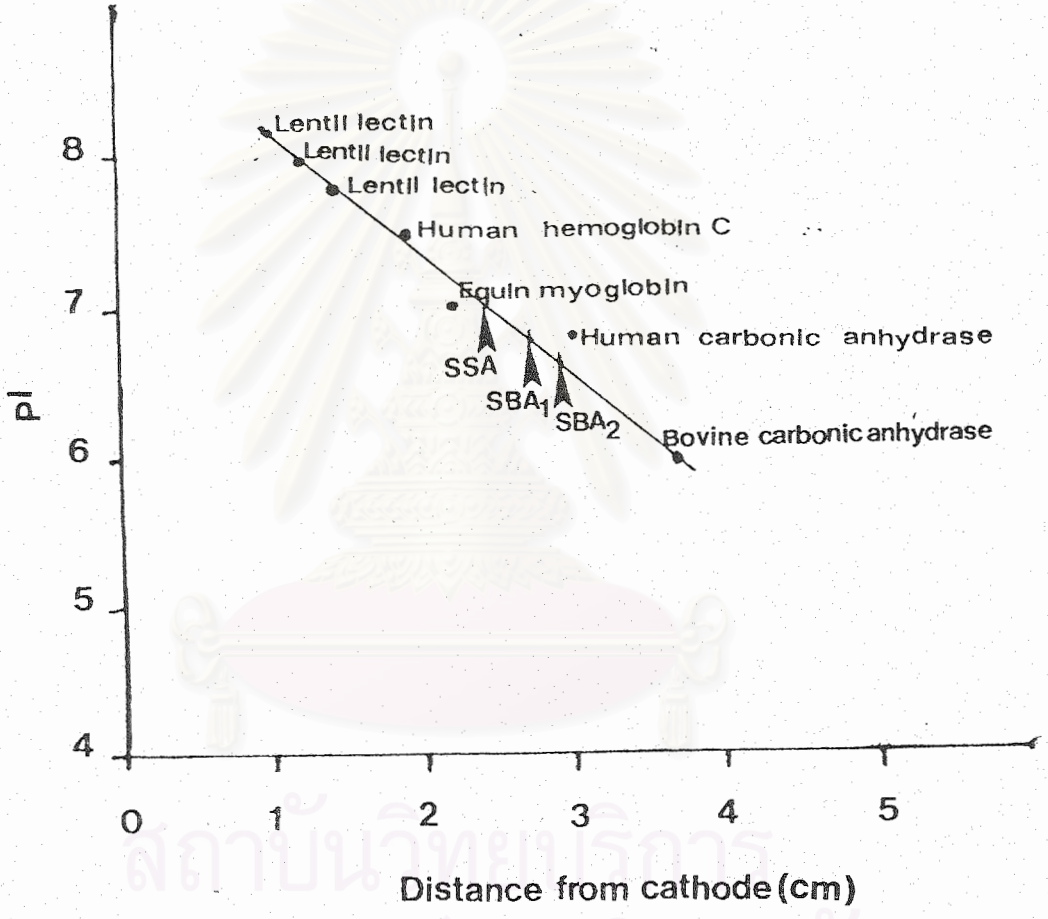


Figure 17 Effect of SSA on Growth of fusarium spp. spores.

A: The spores were germinated in distilled water, magnification 100.

B: The spores were incubated with SSA, magnification 100.

C: Germinating microspore of Fusarium spp., magnification 400.

D: Ungerminating microspores (arrow) of Fusarium spp., magnification 400.

E: Germinating chlamydospore of Fusarium spp., magnification 100.

F: Ungerminating chlamydospore of Fusarium spp., magnification 400.

3.4 Effects of SSA on Plant Pathogenic Fungi

In order to obtain some preliminary data on the possible role of the lectin in nature , tests were carried out on biological effect of SSA on cultured fungi known to be pathogenic to plants especially soybean. Experiments were carried out as described in section 2.2.9.

The observations indicated that SSA from soybean cotyledons was able to inhibit spore germination of all fungi tests as shown in Table 12 and Figure 17-19 . Highest inhibitory effect on spore germination was observed with affinity-purified SSA comparing to other fractions. At the same amount of protein, the lectin affected *Colletotrichum spp*. most (64% inhibition) followed by *Cercospora kikuchii* (58% inhibition) and *Fusarium spp*. (40% inhibition). The inhibition effect was lectin specific since it can be reversed by addition of galactose.

Table 12 Effects of SSA on Spore Germination of Plant Pathogens

Fractions of SSA	% Inhibition		
	Cercospora kikuchii	Colletotrichum spp.	Fusarium spp.
Control (0 mg protein)	3±3	1±1	3±1
Crude extract (0.28 mg protein)	27±2	14±2	24±3
Ammonium sulfate precipitation (0.20 mg protein)	39±1	22±4	35±2
Affinity-purified SSA (0.20 mg protein)	57±2	64±3	39±2
SSA + galactose (0.20 mg protein)	3±3	3±1	3±2

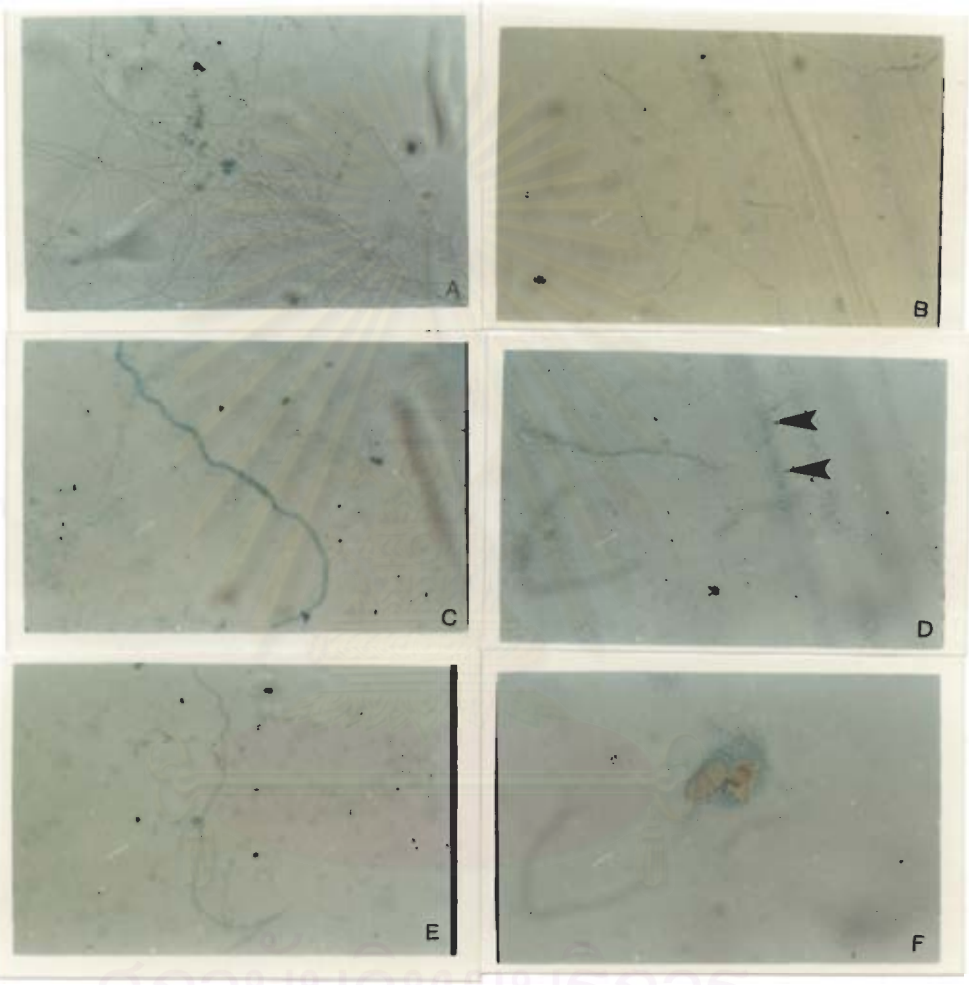


Figure 18 **Effect of SSA on Growth of Cercospora kikuchii spores.**

A: The spores were germinated in distilled water, magnification 100.

B: The spores were incubated with SSA , magnification 100.

C: Germinating spores of Cercospora kikuchii, magnification 400.

D: Ungerminating spores (arrow) of Cercospora kikuchii, magnification 400.

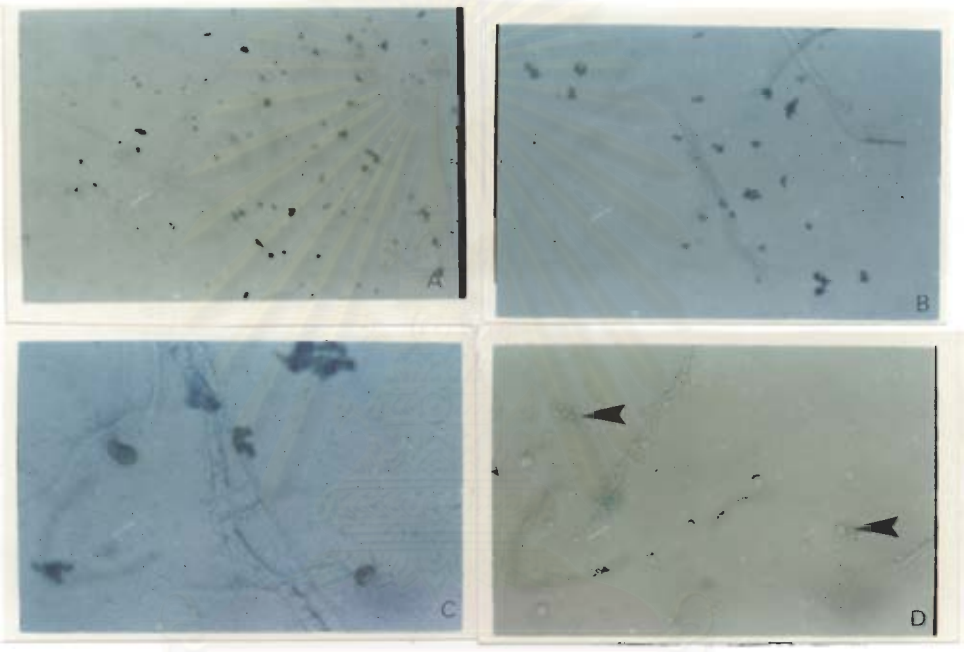


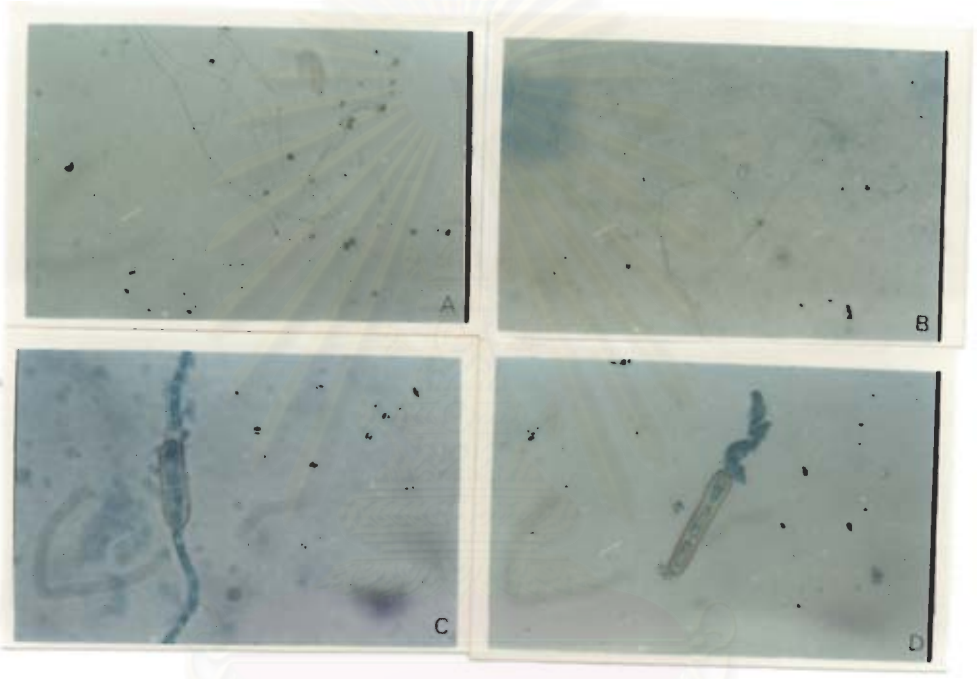
Figure 19 **Effect of SSA on Growth of Colletotrichum spp.** spores

A: The spores were germinated in distilled water, magnification 100.

B: The spores were incubated with SSA, magnification 100.

C: Germinating spore of Colletotrichum spp., magnification 400

D: Ungerminating spore of Colletotrichum spp., magnification 400



3.5 Comparison of Purified SSA with Commercial SBA

Since soybean agglutinin from soybean seed (SBA) has been purified and extensively characterized, it is of interest to investigate whether affinity-purified SSA in this study is similar to the SBA previously reported. SDS-PAGE showed slight difference in the size of the peptide monomer (section 3.3.4) whereas IEF gel showed slight difference (section 3.3.5). Comparision of HA stability of both lectins at varying temperatures and pH's have been reported in section 3.3.1 and 3.3.2. Other properties of SSA and SBA were further investigated and summarized in Table 13.

At equal concentration, SSA and SBA showed similar characteristic on most parameters tested but with different magnitude of effect such as the effect on fungi growth. SSA affects spore germination more than SBA, especially in Collectotrichum spp. HA of SBA doubled that of SSA at the same protein concentration. SBA seems to bind with high affinity to N-acetyl galactosamine while SSA had prefered for galactose. Lactose specificity was observed in SBA only.

Table 13 Comparison of Affinity-Purified SSA with Commercial SBA

Characterization	Commercial SBA (2.00 mg protein)	Affinity-Purified SSA (2.08 mg protein)
-Specific HA activity (U/mg protein)	24,615	12,355
-Sugar specificity (mM/4 HA unit)		
N-acetyl galactosamine	0.01	0.04
Galactose	0.62	0.32
Lactose	0.32	None
-Spore inhibition(%)		
Cercospora kikuchii	39±3	46±4
Colletotrichum spp.	27±2	57±1
Fusarium spp.	32±4	35±1

CHAPTER VI
DISCUSSION

4.1 Lectin in Developing Soybean Seedlings

Lectins in soybean have been reported by several groups of scientists (Table 14). However, the soybean lectins reported were mostly prepared from homogenate of the tissues they were found. Skubatz and Kessler (1984) reported a lectin on the surface of cotyledon of cucumber seedlings which is dependent on the stage of development. Since lectin on surface of soybean has never been reported especially in the seedling, it was of interest to make an investigation. When soybean seeds were germinated and surface soybean seedlings was extracted by immersion of the tissues of interest in distilled water, agglutinin activity was detectable. The extraction of agglutinin from the surface of soybean seedling organs was monitored in the growing seedling for 2 weeks, until the death of the cotyledons and the appearance of the first leaf. The three organs examined; cotyledon, hypocotyl and root, had different amounts of agglutinin released on their surfaces. The release of protein was high in young organs and rapidly decreased with age. The decrease in the amount of surface agglutinin indicated that the agglutinin was not released on the surface at later stage of development (Skubatz and Kessler, 1988).

The results indicate that a lectin is present and easily extracted from the cotyledon surfaces of soybean. A

Table 14 Summary of the Properties of All Lectins in Soybean

Source of soybean agglutinin	Native MW (Dalton)	Properties
Soybean system (seed, leaf, stem, root)(Lotan, 1974)	120,000	-tetrameric of 30,000 -a glycoprotein -specific to Gal and GalNAc -binds specifically to infective strains of Rhizobium japonicum
Lectin released from seed (Fountain, 1977)	30,000	-maximum at 8 hr of seed hydration -specific to Gal or saccharides containing Gal in erythrocyte suspension
Seed (homogenate) (Gade 1981)	56,000-60,000	-Specific to galactose (Gal) and N - acetyl galactosamine(GalNAc) -contained 2 subunits of 28,000 and 30,000 -pI ~ 5.2 by IEF

Table 14 continued

Source of soybean agglutinin	Native MW (Dalton)	Properties
Root (homogenate) (Gade 1981)	84,000-90,000	-contained 3 subunits of 28,000-30,000 and 32,000 -specific to Gal and GalNAc -pI ~ 5.2 by IEF
Seed extract (Agrawal,1984)	-	-affected the development of *Colletotrichum cupisi* and *Fusarium solani*
Commercial seed lectin(from Sigma) (Brambl,1985)	-	-disrupt fungal growth (*Botryodiplodia theobromae*, *Neurospora crassa*, *Aspergillus amslelodami*) by interfering with normal cell wall deposition and assembly
Root (homogenate) Seed (homogenate) Cotyledon (homogenate)	33,000 26,000 and 30,000 16,000 and 30,000	-detect by anti-SBL -seed lectin had 3-4 closely spaced bands on SDS-PAGE

Table 14 continued

Source of soybean agglutinin	Native MW (Dalton)	Properties
Leaf (homogenate) (Vodkin, 1986)	26,000	
Culture soybean cell line SB-1 (Ho,1986)	30,000	-blocked the Rhizobium soybean cell adhesion
SSA (this research)	28,000	-specific to Gal and GalNAc -pI ~ 7.0 by IEF -affected the spore germination of Fusarium spp., Colletotrichum spp. and Cercospora kikushii

few lectins were reported to be extractable with water from surface e.g. in cucumber seedling (Skubatz and Kessler, 1984) and clover roots (Dazzo et al., 1977) or have been located in plant cell walls including epidermal cells (Leach et al.,1982). The very short time required to wash off agglutinin from the cotyledon surface by water (1 minute and even less) showed that the agglutinin probably was present on the epicuticular surface. Only small amount of HA was detected in the cotyledon homogenate after 91 minutes washing.

4.2 Cell and Sugar Specificity of Soybean Agglutinin

Each lectin selectively agglutinated specific type of cells due to the kind of sugars or saccharides present on the cell surface. When tested with red cells from human blood groups and rabbit erythrocytes, the surface agglutinin was specific to human blood group A because there is a lectin recognition site on the red cells containing N - acetyl - D - galactosamine at the end of oligosaccharide structure. The surface agglutinin was specifically inhibited by galactose and N-acetyl galactosamine with the latter showing higher specificity (Table 13), similar to that occured in soybean agglutinin from root and seed studied by Gade et al. (1981). Carbohydrate-binding studies (by inhibition of hemagglutination or glycoprotein precipitation) on the seed soybean lectin (Lis et al.,1970, Pereira et al., 1974) showed the greatest affinity of the SBA for N-

acetyl galactosamine, its glycosides, and oligosaccharides in which this sugar was the nonreducing terminal unit. Rabbit erythrocytes could not be recognized by the surface agglutinin unless treated with trypsin. The treatment of the red cells with the protease may have exposed the sugars on the red cells such that the surface agglutinin binding was possible.

4.3 Purification of the SSA

The purification of surface agglutinin of surface seedling in this work differed from the procedure used by Gade (1981) in two aspects; firstly, Gade did not precipitate the protein with ammonium sulfate. In our experiment, the ammonium sulfate precipitation increased the purity of lectin to 6 folds. Secondly, Conconavalin A column was used by Gade to purifylectin from soybean root and seed, but in this study N-acetyl galactosamine affinity column was used which increased the purity of lectin to 8 folds. The SSA was eluted from the column with galactose because the protein showed affinity to both N-acetyl galactosamine and galactose, the latter was cheaper and readily accessible.

Table 15 A Summary of a Common Characteristics of SSA and SBA

Properties	SSA	SBA
MW Gel filtration	28,000	- *
MW SDS-PAGE	27,500	29,000
pI	7.0	6.8,6.65
Optimum temperature	40	40
Optimum pH	8.0	7.0
Specificity(mM/4HA U)		
N-acetyl galactosamine	0.04	0.01
Galactose	0.32	0.62
Lactose	None	0.32

* Molecular weight of SBA was not determined by gel filtration in this study. A reported value (Lotan,1974) is 120,000.

4.4 Characterization of SSA

Some physicochemical properties of the purified SSA from the affinity column was studied in parallel with purified SBA purcheased from Sigma. Both proteins showed quite similar properties from our study as summarized in Table 15.

Several lectins in soybean have been reported, the properties of which are summarized in Table 14. It can be seen that the molecular weight of most subunits detertmined are in the range of 26,000 - 30,000 Da. Most of the lectins reported were extracted by homogenization of tissues and were reported to exist as a molecule with more than one subunit. There was one report by Fountain (1977) that a lectin with one subunit of 30,000 Da was extracted by hydration of seeds for 8 hours. However, such a long period of seed immersion in water may have swollen the seeds to an extent of cell wall damage and caused a release of intracellular content. In our study, although the native molecular weight of SBA was not determined by gel filtration, its molecular weight on SDS-PAGE was closely similar to SSA (29,000 and 27,500 Da respectively) which was close to the reported value of 30,000 Da for the subunit of SBA. Thus, it seems that SSA from surface of cotyledon may be the same protein as the subunit of SBA reported in the homogenates of seeds and roots of soybean. The native molecular weight of SSA as determined by gel filtration implied that purified SSA may exist as a monomeric molecule. It is possible that, in soybean,

lectin in the seeds and roots all existed as tetramer of a subunit with molecular weight of 28,000-30,000 Da. When the lectin was secreted on the surface such as was found in our study on the surface of the cotyledon, it was in a monomeric form. This is also accountable for some of the different characteristics observed on SSA and SBA such as optimum pH, pI values, degree of binding to sugars and the effect on fungal growth. The commercial SBA appeared on IEF as at least 2 bands and the pI values were 6.8 and 6.65 which were slighly different from that of SSA which was 7.0. The two bands observed for SBA are likely to be two of many isolectins reported previously (see section 1.9).

Considering the observed difference in the affinity forsugars , some speculation could be made. SSA as a single peptide chain may contain binding sites for both N-acetyl galactosamine and galactose, or it may contain one binding site but show different affinity to N-acetyl galactosamine and galactose. Upon association of the monomers to form tetrameric SBA, galactose binding site may be less accessible while binding to N-acetyl galactosamine was somehow enhanced.

4.5 Biological Properties of SSA

When SSA and SBA were tested for their effect on a few species of fungi known to be pathogenic to soybean, they showed varying degree of effect, SSA had greater inhibitory effect on growth of *Cercospora kikuchii* *Colletotrichum spp.*, and *Fusarium spp.* comparing to

SBA. As reported in Table 13 that SSA was more specific to galactose and as shown in Table 12 that inhibitory of SSA on fungal growth can be overcome by addition of galactose, it may be suggested that the interaction of SSA with fungi was at galactose binding site. This also supported with the lower inhibitory effect on fungi observed for SBA. However, this could be confirmed by further experiment on the effect of N-acetyl galactosamine on reversing the inhibition of fungal growth by SSA and SBA.

4.6 Hypothesis on the SSA in Cotyledon of Soybean Seedlings

The appearance of an agglutinin on the cotyledon of soybean seedlings, which seemed to be excreted on the surface due to the ease of extraction by simple immersion in water, implicates some functional importance of the protein. From all the data obtained in comparison to SBA and other reported properties, a hypothesis on the role of the lectin is postulated. The existance of SSA may be a product of genetic polymorphism as reviewed in section 1.6.1. In the soybean Glycine max, there may exist several homologous genes encoded for soybean agglutinins. Lectins in seed and root of soybean have already been reported (Table 14). In our study, SSA was found on the cotyledon surface of 3-day old seedlings. This may be the example case of a developmentally regulated expression of homologous gene. Gene for SSA may be expressed at the time of seed germination into seedlings and its product is a peptide monomer with molecular weight of 28,000-30,000 Da

which is secreted as such on the surface. It may have primary function to prevent growth of pathogenic fungi, as one step of defense mechanism of the plant. On the other hand, SBA which was in tetrameric form and exists in the seed may either be the storage form of soybean in the seed and dissociates into subunit and excreted as such to the surface. The report by Fountain (1977) that a lectin of molecular weight 30,000 was released from seed upon immersion for 8 hours in water is one supporting evidence for our hypothesis. Perhaps, SSA and SBA may the products of the same gene but they expressed in different stage of development or have different transcription product so SSA and SBA were differed in some asspects. Otherwise, SBA itself may have other role(s) which has yet to be identified.

SUMMARY

1. Soybean surface agglutinin (SSA) was extracted from cotyledons of 3 - day old soybean seedlings. The SSA was specific to N-acetyl galactosamine and galactose. It was purified in two steps i.e. ammonium sulfate fractionation and affinity column.

2. Several properties of SSA were investigated in parallel with commercial soybean agglutinin (SBA) ,Its molecular weight determined by gel filtration and SDS-PAGE was 28,000 indicating its existence as a monomeric molecule. Its pI was 7.0 as determined by IEF gel. Its hemagglutination activity was optimum at pH 8.0 and the optimum temperature was 40 °C. Most of these properties were similar to SBA purified from soybean seed with slight difference in some aspects.

3. The SSA can inhibit the germination of spores of some soybean pathogenic fungi.

REFERENCES

Agrawal, P. and Maahadevan, A., (1984). " The Lectins in Germinating Seeds and Their Effect on Fungi", Acta Phytopathol. Acad. Sci. Hung., 19: 219-236

Allen, A. K., Neuberger, A.,(1975). " A Simple Method for the Preparation of an Affinity Absorbent for Soybean Agglutinin Using Galactosamine and CH Sepharose", FEBS Lett., 50: 362-364

Barkai-Golan, R., Mirelman, D., Sheeran, N.,(1978)." Studies and Growth Inhibition by Lectins of Penicillia and Aspergilli", Arch. Microbial., 116: 119-124

Basha, S M. M., and Robert, R. M.,(1981)." The Glycoproteins of Plant Seeds", Plant Physiol., 67: 936-939

Baumann, C. M., Rudiger, H., and Stronsberg, A. D., (1979). " A Comparison of the Two Lectins from Vicia cracca ", FEBS Lett., 102: 216-218

_____, C. M., and Rudiger, H.,(1981)." Interaction Between the Two Lectins from Vicia cracca", FEBS Lett., 136: 279-283

Bohlool B. B., Schmidt, E. L.,(1974)." Lectins: A Possible Basis for Specificity in the Rhizobium-legume Root Nodule Symbiosis", Science, 185: 269-271

_____, C. M., Schmidt, E. L.,(1976)."Immunofluorescent Polar Tips of Rhizobium japonicum: Possible Site of Attachment or Lectin Binding", J. Bacteriol, 125: 1188-1194

Borrebaeck, C. A. K. and Mattiasson, B.,(1983)." Distribution of a Lectin in Tissues of Phaseolus vulgaris",

Physiol. Plant., 58: 29-32

Bowles, D. J., and Marcus, S.,(1981)." Characterization of Receptors for The Endogenous Lectins of Soybean and Jackbean Seeds", FEBS Lett., 129: 135-138

Boyd, W. C., and Shapleigh, E.,(1954)."Specific Precipitating Activity of Plant Agglitinins (Lectins)", Science, 119: 419

Brambl, R. and Gade, W., (1985). " Plant Seed Lectins Disrupt Growth of Germinating Fungal Spores ", Physiol. Plant, 64: 402-408

Calvert, H. E., Lalonde, M., Bhuvaneswari, T.V. and Bauer, W.D., (1978). " Role of Lectins in Plant Microorganism Intertactions. IV. Ultrastructural Localization of Soybean Lectin Binding Sites on Rhizobium japonicum",Can.J.Microbial., 24: 785-793

Dazzo, F. B. and Hubbell, D. H.,(1975)." Cross-reactive Antigens and Lectin as Determinants of Symbiotic Specificity in Rhizobium - Clover Association" Appl. Microbiol., 30: 1017-1033

_____,F. B., Napoli, D. H. and Hubbell, D. H.,(1976) " Adsorption of Bacteria to Roots as Relate to Host Specificity in the Rhizobium - Clover Symbiosis", Appl. Environ. Microbiol., 32: 168-171

_____,F. B., Brill, W. J.,(1977). " Receptor Site on Clover and Alfalfa Roots for Rhizobium" Appl. Environ. Microbiol., 33: 132-136

_____,F. B., and Brill, W., (1987). " Recognition by Fixed Nitrogen of Host-symbiont Recognition in Rhizobium - Clover Symbiosis", Plant Physiol.,

62: 18-21

Fountain, D. W., Foard, D. E., Replogle, W. D. and Young, W. K., (1977) " Lectin Release by Soybean Seeds", Science, 197: 1185-1187

Gade, W., Jack, M. A., Dahl, J. B., Schmidt, E. L., Wold, F., (1981)." The Isolation and Characterization of a Root Lectin From Soybean (Glycine max(L) Cultiva cheppewa)",J. Biol. Chem., 256:12905-12910

Gansera, R., Schurz, R. and Rudiger, H., (1979)." Lectin Associated Proteins from the Seeds of Leguminosae", Hoppe-Seyler's Z. Physiol. Chem., 360: 1579-1585

Gatehouse, J. A. and Boulter, D.,(1980)." Isolation and Properties of a Lectin From the Roots of Pisum sativum", Physiol. Plant, 49: 437-442

Gebauer, G., Schlitz, A., Schimpl, A. and Rudiger, H., (1979)." Purification and Characterization of a Mitogenic Lectin and a Lectin-binding Protein from Vicia sativa",Hoppe-Seyler's Z. Physiol. Chem. 360: 1727-1735

_____, G., Schimpl, A. and Rudiger, H.,(1982)." Lectin Binding Proteins as Potent Mitogens from nu/nu Mice", Eur. J. Immunol., 12: 491-495

Gibson, D. M., Stack, S., Krell, K. and House, J.,(1982)." A Comparison of Soybean Agglutinin In Cultivars Resistant and Susceptible to Phytophthora wegasperma var sojae (Race 1)", Plant Physiol., 70: 560-566

Goldstein,I. J., (1972). In Methods of Carbohydrate

Chemistry , (R. L., Whistler and J. N. Be Miller, eds.), vol. II: 106-119, Academic press, New York

_____, I. J., and Hayes, C. E.,(1978)." The lectins: Carbohydrate Binding Proteins of Plants and Animals", Adv. Carbohyd. Biochem., 35: 127-340

_____, I. J., Hughes, R. C., Monsigny, M. Osawa, T. and Sharon, N.,(1980). " What Should Be Called a Lectin?", Nature, 285: 66

Gordon, J. A., Blumberg, S., Lis, H., and Sharon, N.,(1972). " Purification of Soybean Agglutinin by Affinity Chromatography on Sepharose-N-=∈-aminocaproyl-α-D-galactopylano Sylamine", FEBS Lett., 24: 193-196

Hapner, K. D. and Robbins, J. E.,(1979). " Isolation and Properties of a Lectin from Sainfoin (Onobrychis viciifolia)", Biochim. Biophys. Acta,580: 186-197

Ho, S. C., Malek-Hedayat, S., Wang, J. L. and Schindler, M., (1986) " Endogenous Lectins from Cultured Soybean Cells: Isolations of a Protein Immunologically Cross-reactive with Seed soybean Agglutinin and Analysis of Its Role in Binding of Rhizobium japonicum " The Journal of Cell Biology, 103: 1043-1054

Horejsi, V. and Kocourek, J.,(1974b).," Phytohemagglutinins. XVII. Properties of the Anti-H Specific Phytohemagglutinin of Furze Seed (Ulex europaeus)", Biochim. Biophys. Acta, 336: 338-343

_____, V., Ticha, M. and Kocourex, J., (1979). " Affinity Electrophoresis", Trends Biol. Sci., 4: N6-N7

Howard, I. K., Sage, H. J. and Horton, C. B.,(1972)."Studies on the Appearance and Location of Hemagglutinins from a Commom Lentil During the Life Cycle of the Plant" Arch. Biochem. Biophys., 149: 323-326

Igiesias, J. L. O., Lis, H. and Sharon, N. (1982). " Purification and Properties of a D-galactose/=N-acetyl-D-galactosamine Specific Lectin ", Eur. J. Biochem., 123: 247-252

Jaffe, J. G., (1980). " Hemagglutinins ", pages 73-102 in Toxic Constituents of Plant Food-stuffs, 2nd ed.

Janzen, D. H., Juster, H. B. and Liener, I. E., (1976). "Insecticidal Action of the Phytohemagglutinin in Black Beans on a bruchid Beetle", Science, 192: 795-796

_____, D. H., (1981). " Lectins and Plant-herbivore Interaction", Recent Adv. Phytochem., 15: 241-258

Kijne, H. W., van der Schaal, I. A. M. and De Vries, G. E., (1980). " Pea Lectins and the Recognition of Rhizobium leguminosarum" Plant Sci., 18: 65-74

Kornfeld, S. and Kornfeld, R., (1978). In The Glycoconjugates, (M. I. Horowitz and W. Pigman eds.) Vol II, pp. 339-384. Academic Press.

Kristiansen, T., (1974). vol 34:"Affinity Techniques: Enzyme Purification, part B" Method in Enzymology, (W. B. Jakoby and M. Wilchek, eds.) vol 34:331-341. Academic press, New York

Laemli, U. K.,(1970)." Cleavage of Struture Protein During the Assembly of the Head of Bacterio Phage", Nature, 227: 680-685

Law, I. J. and Strijdom, B. W., (1982). " Lotononis bainesii Seed and Root Lectins and the Interaction with Rhizobium", S. Afr. J. Sci., 78: 375

Leach, J. E., Cantrell, M. A. and Sequeira, L., (1982). " A Hydroxyproline - rich Bacterial Agglutinin from Potato : Its Location by Immunofluorescence " Physiol Plant Pathol., 21: 319-325

Liener, I. E., (1975). " Oucurence of Methionine Sulfoxide in Potato Tubers After Acid Hydrolysis" Anal. Biochem, 68: 651-653

_____,I. E., (1981). " The Nutritional Significance of Plant Lectins",Antinutrients and Natural Toxicants in Foods,(R. L. Ory, ed), pp. 143-157

Lis, H., Sharon, N. and Katchalski, E.,(1966)." Soybean Hemagglutinin, a Plant Glycoprotein.I. Isolation of a Glycopeptide", J. Biol. Chem., 241: 684-689

_____, H., Sela, B. A., Sachs, L. and Sharon, N., (1970). "Specific Inhibition by N-acetyl-D- galactosamine of the Interaction Between Soybean Agglutinin and Animal Cell Surfaces", Biochim. Biophys. Acta., 211: 582-585

_____,H. and Sharon, N., (1973). " The Biochemistry of Plant Lectins (Phytohemagglutinins)", Annu. Rev. Biochem. 42: 541-574

_____, H., Lotan, R. and Sharon, N., (1974) " Synthesis and Use of Affinity Chromatography Columns for the Purification of Plant Lectins", Ann. NY. Acad. Sci., 234: 232-238

_____, H. and Sharon, N., (1977)."Lectins: Their Chemistry and Application to Immunology", In The Antigens, (M. Sela, ed.), 4: 429-529, New york: Academic

_____,H. and Sharon, N., (1981). " Lectins in Higher Plants", In The Biochemistry of Plants (P. K. Stumpf and E. E. Conn, Eds.) Vol. 6: 371-447 Academic Press,Inc., New York.

Lotan, R., Siegelman, H. W., Lis, H. and Sharon, N. (1974) " Subunit Structure of Soybean Agglutinin", J. Biol. Chem., 249: 1219-1224

Lowry, P. H., Rosenbouge, N. J., Farr, A. L. and Randall, P. J., (1951). " Protein Measurement with the Folin Phenol Reagent", J. Biol. Chem., 193: 265-275

Naca, R. D. and Hoak, J. C., (1974). " Endotherial Injury and Platelet Aggregation Associated With Acute Lipid Mobilization", J. Natl. Cancer Inst., 52: 365-367

Marikovsky, Y., Lotan, R., Lis, H., Sharon, N. and Dannon. D. (1976). " Agglutination and Labeling Density of Soybean Agglutinin on Young and Old Human Red Blood Cells", Exp. Cell Res., 99: 453-456

Miller, R. C. and Bowles, D. J., (1982) " A Comparative Study of the Localization of Wheat Germ Agglutinin and Its Potential Receptors in Wheat Grain" Biochem. J., 206: 571-576

Mirelman, D., Galun, E., Sharon, N. and Lotan, R., (1975). " Inhibition of Fungal Growth by Wheat Germ Agglutinin" Nature, 256: 414-416

Neely, D. and Himelick, E. B., (1966). " Simutaneous

Determination of Fungistatic and Fungisidal Properties of Chemicals", Phytopathology, 56: 203-209

Neurohr, K. J., Young, N. M., Smiyh, I. C. P. and Mountsch, H. H., (1981). " Kinetics of Binding of Methyl - and -D-galactopyranoside to Peanut Agglutinin: A Carbon-13 Neuclear Magnetic Resonamce Study", Biochemistry, 20: 3499-3504

Orf, J. H., Hymowitz, T., Pull, S. P. and Pueppke, S. G., (1979). " Inheritance of a Soybean Root Lectin", Crop. Sci., 18: 899-900

Pueppke, S. G., Bauer, W. D., Keegstra, K. and Ferguson, A. L., (1978). " Distribution of Soybean Lectin in Tissues of Glycine max(L)", Plant Physiol., 61: 779-784

Pull, S. P., Pueppke, S. G., Hymowitz, T. and Orf, J. H., (1978). " Soybean Lines Lacking the 120,000 Dalton Lectin", Science, 200: 1277-1279

Pusztai, A., Grant, G. and Stewart, J. C., (1981). " A New Type of Phaseolus valgaris (c.v. Pinto III) Seed Lectin: Isolation and Characterization", Biochim. Biophys. Acta, 671: 146-154

Quiochio, L. F. (1986). " Carbohydrate-binding Proteins: Tertiary Structurs and Protein-sugar Interactions", Ann. Rev. Biochem., 55: 287-315

Rouge, P., and Pere, D., (1982). In Lectins, Biology, Chemistry, Chemical Biochemistry, (Bog-Hansen, T. C. ed.) De Gruyter, Burlin, 2: 137-150

Rudiger, H., (1984). " On the Physiological Role of Plant

Lectins", 34: 95-99

Schurz, H. (1982). " Ein Lectinbindendes Protein aus Pisum sativum Isolierrung, Charakterisierung und Wechselwiskung mit dem Lectin" Thesis, Wiirzburg University, Wurzburg, West Germany.

Sequeira, L., (1978). " Lectins and Their Role in Host - pathogen Specificity ", Annu. Rev. Phytopathol., 16: 453-481

Sharon, N. and Lis, H., (1972) " Lectins: Cell-Agglutinating and Sugar-specific Proteins", Science, 177: 949-959

_____, N., (1979). In Glycoconjugate Research. (J. D. Gregory and R. W. Jeanloz, eds.) Vol I, pp. 459-491

Sing, V. O. and Schroth, M. N. (1977). " Bacteria-plant Cell Surface Interactions: Active Immobilization of Saprophytic Bacteria in Plant Leaves" Science, 197: 199-206

Skubatz, H. and Kessler, B., (1984) " A Development-Dependent Hemagglutinin from Cucumbert Surface" Plant Physiol., 76: 55-58

_____, H. and Kessler, B., (1988). " Age-dependent Apperance of Specific Proteins on Cucumber Surface Under Normal Growth Condition", Plant Science, 56: 27-30

Strosberg, A. D., Buffard, D., Lauwereys, M. and Forris, A., (1986). " Legume Lectins: A Large Family of Homologous Proteins ", In The Lectins: Properties, Functions, and Applications in Biology and

Medicine, (I. E. Liener, N. Sharon and I. J. Goldstein, eds.), pp. 249-264, Academic Press.

Surolia, A., Bachhowat, B. K. and Podder, K., (1975). " Interaction of Immobilized Lectin from Ricinous communis with a Simple Sugar and a Polysaccharide" , Nature, 257: 802-804

Talbot, C. F. and Etzler, M. E., (1978 b). " Isolation and Characterization of a Protein from Leaves and Stems of Dolichos biflorus that Crossreacts With Antibodies to the Seed Lectin" Biochemistry, 17: 1474-1479

Vodkin, L. O., Rhodes, P. R. and Goldberg, R. B., (1983) " A Lectin Gene Insertion has the Structural Features of a Transposable Element" Cell, 34: 1023-1031

_____, L. O. and Raikhel, N. V., (1986). " Soybean Lectin and Related Proteins in Seeds and Roots of Le and Le Soybean Varieties", Plant Physiol., 81: 558-565

Wada, S., Pallinsch, M. J. and Liener, I. E., (1958). " Chemical Composition and End Groups of the Soybean Hemagglutinin" J. Biol. Chem., 233: 395-400

Watkins, W. M., (1966) " Blood Group Specific Substances" In Glycoproteins (A. Gottschalk, ed.) pp. 467-515. Elsevier Publishing Company.

Wolpert, J. S., Albersheim, P., (1976). " Host-Symbiont interaction I. the lectins of legumes Interact with the O-antigen-containing lipopolysaccharides

of their symbiont Rhizobia", Biochem. Biophys. Res. Commu., 70: 729-737

Zatta, P. F. and Cummings, R. D., (1992). " Lectins and their Uses as Biotechnological Tools", Biochemical Education, 20: 2-9

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