

LECTINS IN THAI PLANTS : PURIFICATION AND CHARACTERIZATION
OF A LECTIN FROM JACK FRUIT (*Artocarpus heterophyllus*)

BY

PISIT NAMJUNTRA

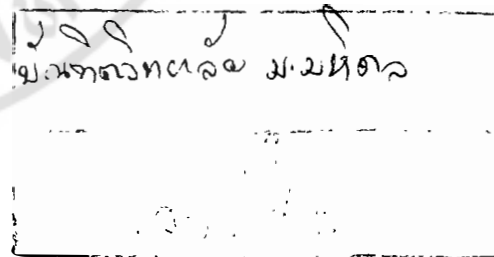
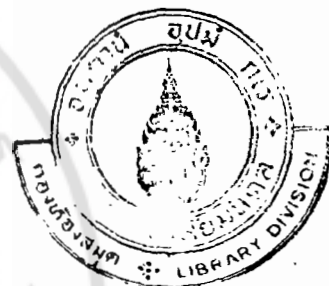
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This thesis

entitled

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was submitted to the Faculty of Graduate Studies, Mahidol University
for the degree of Master of Science on September 3, 1984

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OF A LECTIN FROM JACK FRUIT (*Artocarpus heterophyllus*)

We further agree that he has satisfactorily defended his
thesis at the examination given by the Supervisory Committee

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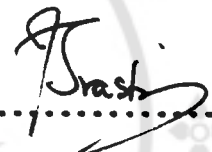
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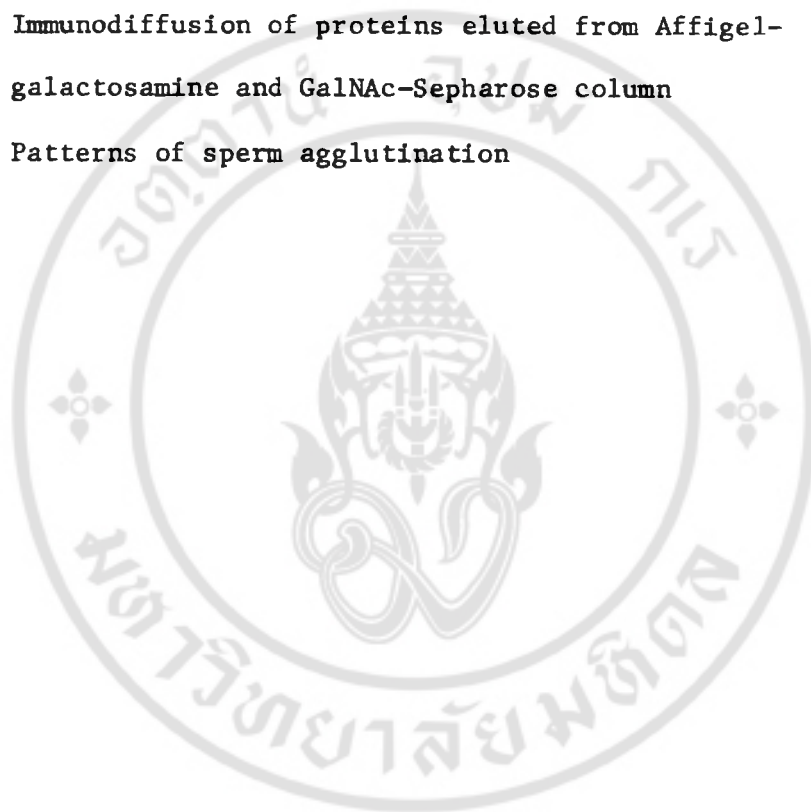
LIST OF ABBRVIATIONS

ConA	Concanavalin A
EDTA	Ethylenediamine tetraacetate
Gal	Galactose
GalNAc	N-acetylgalactosamine
h	Hour
HRBC	Human red blood cells
M	Molar
mA	Milliampere
mM	Millimolar
MW	Molecular weight
ND-PAGE	Non-denaturing polyacrylamide gel electrophoresis
nm	Nanometer
PBS	Phosphate buffer saline
PHA	<i>Phaseolus vulgaris</i> phytohemagglutinin
PNA	Peanut agglutinin
RCd	Rat cauda epididymal sperm
RCp	Rat caput epididymal sperm
SDS	Sodium dodecylsulfate
SDS-PAGE	Sodium dodecylsulfate polyacrylamide gel electrophoresis
TEMED	N,N,N',N'-tetramethylene ethylenediamine
Tris	Tris-(hydroxymethyl)-aminomethane
UHS	Unwashed human sperm
WHS	Washed human sperm
WGA	Wheat germ agglutinin

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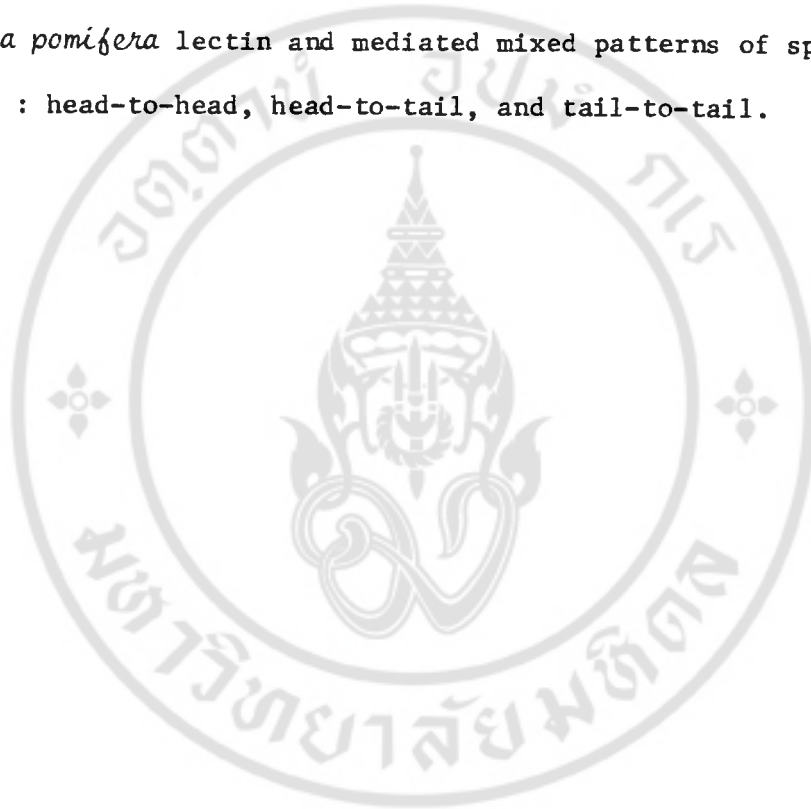
ABSTRACT

The seeds of Thai plants were screened for new lectins using hemagglutination and sperm agglutination tests. Among thirty-one kinds of seeds, one kind of storage root, and one kind of stem tested, fifteen kinds of seeds showed agglutinating activities. Most of the active seed extracts were from family Leguminosae. Other active seed extracts were from family Moraceae, Sapindaceae, Gramineae, and Cucurbitaceae.

Seed extracts from Jack fruit, red kidney bean, broad bean, and garden pea agglutinated rat sperm, human sperm and human erythrocytes. Sa-taw, soybean, and yard-long bean seed extracts agglutinated only rat and human sperm but not human erythrocytes. Seed extract from rambutan agglutinated rat caput epididymal sperm and human erythrocytes while seed extract from longan reacted only with caput epididymal sperm. The extract from tamarind agglutinated rat sperm and human erythrocytes but not human sperm. Seed extract from balsam pear agglutinated only human red blood cells.

Jack fruit lectin was purified from the crude seed extract by ammonium sulfate fractionation followed by affinity chromatography on a column of Affigel-galactosamine. The purified Jack fruit lectin was sensitive to pronase and bound specifically to N-acetylgalactosamine. By gel filtration, the molecular weight of the native lectin was 42,000. By SDS-PAGE, the lectin was found to compose of two dissimilar subunits with molecular weight of 18,000 and 13,000. The

small subunit existed in greater amount than the large one. A trimeric structure consisting of two small and one large subunits was proposed as the structure of the Jack fruit lectin. By non-denaturing polyacrylamide gel electrophoresis, the lectin showed multiple forms with charge heterogeneity. Jack fruit lectin also cross-reacted with anti-*Maclura pomifera* lectin and mediated mixed patterns of sperm agglutination : head-to-head, head-to-tail, and tail-to-tail.



1. INTRODUCTION

1.1 General properties of lectins

Lectins are carbohydrate-binding proteins or glycoproteins of non-immune origins that can precipitate glycoconjugates and/or agglutinate cells. Initially, they were found in plants, mostly in legumes. To date, several lectins from various sources, plants and animals, have been purified and characterized. Lectins are similar in many respect to antibodies. The difference between lectins and antibodies is that antibodies are produced by the immune system of animals against antigenic stimulus while lectins are formed presumably without the requirement of such a stimulus and can be found in organisms lacking immunologic responses. Lectins are not enzymes since they devoid of enzymatic activity (Kocoureck and Horejsi, 1981). They can bind specifically with sugars, mono- or oligo-saccharides. They bear at least two carbohydrate binding sites so they can be cross-linking agents between the cells or glycoconjugates. Their ability to agglutinate cells are useful in study of cell surface membrane and change of membrane. Lectins bound to the carbohydrate-containing groups on cell surface can be detected by biochemical or microscopic methods. Some lectins can agglutinate transformed cells but not normal cells. Many lectins have been employed to demonstrated significant change of cell membrane during maturation, for instance, the surface change of sperm cells (Reviewed in Koehler, 1978).

Lectins were first identified in extracts of plant seeds that were found to contain factors that agglutinate red blood cells. These factors were called "phytohaemagglutinin" or "hemagglutinin" (for review see Sharon and Lis, 1972; Lis and Sharon, 1973; Nicolson, 1974; Lis and Sharon, 1977; Brown and Hunt, 1978). The word "lectin" (Latin, to select or choose) was first proposed in the year 1954 by Boyd and Sharpless (see Lis and Sharon, 1977) to include hemagglutinins showing selectivity in their agglutination of erythrocytes of different animal species, and some showing human blood-type specificity. The hemagglutinating activity of plant extracts was first observed in the year 1888 by Stillmark (see Lis and Sharon, 1977) who studied *Ricinus communis* (castor bean) agglutinin. Stillmark found some selectivity in lectin agglutination of red cells from different animals. This observation was further extended by Landsteiner's finding (see Brown and Hunt, 1978) that lectins did not agglutinate all red cells to the same extent and the agglutination was dependent on the species and blood type of the donor. A number of hemagglutinins were later shown to be specific in their reactions with red cells of human blood group ABO and MN (see Pusztai et al., 1983). The differential agglutination of red cells based on blood type clearly indicated that different lectins must bind to different chemical groups on the red cell membrane. The identity of the group recognized by a given lectin is ordinarily determined by measuring the ability of mono- or oligosaccharides to inhibit lectin-induced hemagglutination. For instance, lectins specific to blood group A was inhibited by N-acetyl-D-glucosamine, while those specific for blood group O were inhibited by α -methyl-L-fucose (Morgan and Watkins, 1959). Table 1 shows some of

the lectins with blood group-specificity and their corresponding inhibitory sugars.

In sugar inhibition test, simple sugars and glycosides are almost as potent as complete oligosaccharides, but in most cases the complex oligosaccharides are several thousandfold more potent than simple sugars (Kornfeld and Kornfeld, 1978). For example, the blood group A-reactive hemagglutinin from the snail *Helix pomatia* was inhibited to a somewhat greater extent by methyl- α -D-GalNAc than the A-active pentasaccharide (Hammerstrom and Kabat, 1969) whereas the erythro-agglutinating phytohemagglutinin (PHA) from *Phaseolus vulgaris* is inhibited more than 60,000 times better by a glycopeptide derived from human erythrocyte membranes (Fig. 1) than by the simple sugars contained in the glycopeptide (Kornfeld and Kornfeld, 1971).

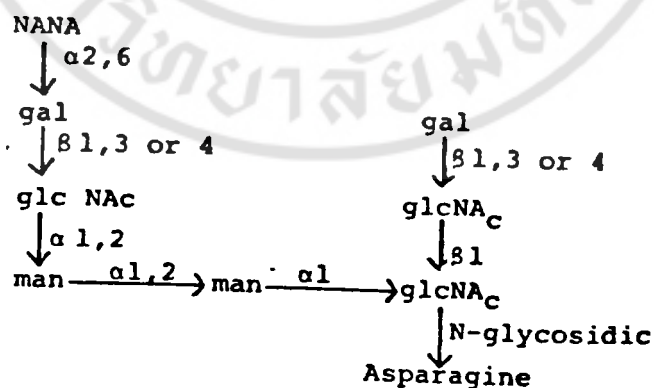


Fig. 1 Structure of the oligosaccharide units of the human erythrocyte trypsin-released glycopeptides (from Kornfeld and Kornfeld, 1971).

TABLE 1 Blood group-specific lectins and inhibitory sugars
(taken from Brown and Hunt, 1978)

<u>Blood group</u>	<u>Lectin source</u>	<u>Inhibitory sugars</u>
A	<i>Crotolaria aegyptica</i>	D-gal
	<i>Dolichos biflorus</i> (horse gram)	D-galNAc
	<i>Phaseolus lunatus</i> (lima bean)	D-galNAc
	<i>Vicia cracca</i>	D-galNAc
	<i>Helix pomatia</i> (garden snail)	D-galNAc
A + B	<i>Sophora japonica</i>	D-gal, D-galNAc
	<i>Calpurina aurea</i>	-
	<i>Coronilla varia</i>	-
B	<i>Bandeiraea simplicifolia</i>	D-gal
	<i>Marasmius oreades</i>	-
	<i>Polyporus fomentarius</i>	-
O	<i>Anguilla anguilla</i>	L-fuc
	<i>Cystisus sessilifolius</i>	(GlcNAc) ₂
	<i>Lotus tetragonolobus</i>	L-fuc
	<i>Ulex europeus</i>	L-fuc
M	<i>Iberia amava</i>	-
N	<i>Vicia graminia</i>	-

1.2 Sources of lectins

Lectins have been extracted mostly from plants, especially within the Leguminosae. According to Toms and Western (1971) about 95% of the lectins reported since 1948 were from the seeds of legumes. Apart from this, lectins have also been isolated and purified from bacteria, slime molds and animals. In several sources, lectins do not occur singly, but as a group of closely related proteins, or isolectins, with very similar chemical and biological properties. A list of organisms that contain lectins is shown in Table 2.

1.3 Location of plant lectins

In plants, agglutinins are present mainly in those parts of plants which contain reserve nutrients and they disappear from these tissues at the same time as the stored reserve proteins (Eisler and von Portheim, 1911; see Pusztai et al., 1983). However, there are a number of notable exceptions known. For example, in wheat grains the lectin is restricted to the germ and after germination the lectin level remains high for at least 34 days (Pusztai et al., 1983). Lectins can also be found in leaves, stems, and hypocotyls. In *Phytolacca americana* most of lectins are found in leaves and stems of the plant (Farnes et al., 1964). It is generally agreed that Leguminosae lectins, the best-studied group, are preferentially localized in the cotyledons (Rudiger, 1984). This has been confirmed by the finding of *Phaseolus vulgaris* lectin (Barker et al., 1976), soy bean lectin (Horisberger and Rosset, 1977) and *Datura stramonium* lectin (Kilpatrick et al., 1979) in protein bodies of the cotyledon. Protein bodies are organelles of 2 to 3 μm diameter, surrounded by a unit membrane (Higgins et al., 1983).

TABLE 2 Sources of lectins (from Lis and Sharon, 1977)Plants-legumes*Arachis hypogaea* (peanut)*Canavalia ensiformis* (jack bean)*Dolichos biflorus* (horse gram)*Glycine max* (soy bean)*Lens curinaris* (lentil)*Lotus tetragonolobus* (winged bean)*Phaseolus vulgaris* (red kidney bean)*Phaseolus lunatus* (lima bean)*Pisum sativum* (garden pea)*Vicia cracca**Vicia faba* (broad bean)*Vicia gramineae*Plants-others*Phytolacca americana* (pokeweed)*Solanum tuberosum* (potato)*Triticum vulgare* (wheat)*Wistaria floribunda*Bacteria*Pseudomonas aeruginosa*Slime mold*Dictyosteleum discoidium**Dictyosteleum purpureum*Higher animals

Rabbit

Lower animals*Anguilla anguilla* (eel)*Helix pomatia* (garden snail)*Limulus polyphemus* (horseshoe crab)*Geodia cydonium* (sponges)

They contain several storage proteins. Protein deposition in protein bodies occurs during the maturation of seeds and this process is reversed in germination.

In cereals, the plants in Family Gramineae, lectins have been reported in the embryo. Wheat germ agglutinin (WGA) is localized in peripheral portions of wheat embryo, that is, in the root cap, coleorhiza, coleoptile, and scutellum while barley lectin is present only at the periphery of embryonic roots (Mishkind et al., 1983). Mishkind et al (1983) concluded that organs that become externalized during germination of the embryo tend to contain lectins, at least at the periphery.

Extracts from cell wall of plants have been reported to contain hemagglutinating substances. Haass et al (1977) have extracted protein from cell walls of mung bean hypocotyls and detected the hemagglutination activity. Langston and Gade (1982) have also isolated a lectin specific for 1,3- β -glucans from cell wall of oats (*Avena sativa*).

1.4 Methods of isolation

Lectins can be isolated by conventional protein fractionation. However, the best and most convenient method is affinity chromatography. In this technique, the lectin is adsorbed to a suitable immobilized adsorbent and then eluted with specific sugar. Agrawal and Goldstein (1967) used commercial available dextran, Sephadex G-50, to purify Concanavalin A (Con A). In other instances, various saccharides and lectin-binding glycoproteins are coupled to supporting matrix to form affinity column (Lis et al., 1974). For example, thyroglobulin

has been coupled to Sepharose to use as adsorbent in purification of *Phaseolus vulgaris* lectin. (Felsted et al., 1975). Several coupled adsorbents are now commercially available, for example, ConA-Sepharose, lentil lectin Sepharose, WGA-Sepharose, GlcNAc-Sepharose and GalNAc-Sepharose.

1.5 Molecular structure of lectins

Lectins seem to have no structural features common to all, except that they are proteins. They vary in composition, molecular weight, subunit structure, and number of sugar-binding sites per molecule. Most of lectins are glycoproteins, such as soy bean lectin, pea lectin, *Phaseolus vulgaris* lectin, however some lectins are simple proteins, for example ConA, WGA, and peanut agglutinin. Most legume lectins are poor in sulfur containing amino acids and generally devoid of cysteine (Roberts and Goldstein, 1983) while some lectins, e.g., potato lectin and pokeweed mitogen (lectin) are rich in cysteine (Lis and Sharon, 1977). The molecular weight of lectins range from 10,000 (for *Anguilla rostrata* lectin; Barondes, 1984) to several hundred thousands, e.g., 420,000 for horseshoe crab lectin (Lis and Sharon, 1970).

All lectins are multimeric molecules; the number of subunits per molecule varies markedly. For instance, WGA is composed of two subunits while garden snail lectin and horseshoe crab lectins are composed of 6 and 18 subunits, respectively. The subunits may be either identical or nonidentical. For example, the subunits of *Maclura pomifera* lectin differ slightly in their molecular weight, 12,000 and 10,000 (Bausch and Poretz, 1977). In the case of *Griffonia*

simplicifolia leaf lectin GS II, the subunits are different markedly in MW : 33,000 and 19,000 for the two types of subunits (Lamb et al., 1983). There is usually one carbohydrate-binding site per subunit. However, some lectins, for example WGA bear two carbohydrate-binding sites per subunit (Lis and Sharon, 1977).

As mentioned above, lectins are specific to mono- or oligosaccharides. Thus, the binding sites of lectins vary in size, corresponding to that of a single monosaccharide or oligosaccharide (Lis and Sharon, 1977). Knowledge of the sugar specificity of lectins is based on hapten-inhibition studies. Various saccharides were tested for their ability to inhibit lectin-induced agglutination of precipitation, usually hemagglutination which is the conventional method to assay lectin agglutination activity.

Lectins are usually metalloproteins containing predominantly the divalent cations Ca^{2+} , Mn^{2+} , and Mg^{2+} (Lonnerdal et al., 1983). They require metal ions for their activities. For example, in the case of ConA, a transition metal ions must bind to one site in order for Ca^{2+} to bind to another site and not until both sites are occupied will ConA bind carbohydrate (Kalb and Levitzki, 1968)

Lima bean lectin also requires Ca^{2+} and Mn^{2+} for carbohydrate binding activity (Roberts and Goldstein, 1984). *Dolichos biflorus* lectin binds several divalent cations but only Ca^{2+} is required for the lectin to express full binding activity (Borrebaeck et al., 1981). Lonnerdal et al (1983) have shown that binding activities of eighteen different lectins depend on their metal ion contents: when the cations were removed the lectins lost their carbohydrate binding activity.

1.6 Interaction of lectin with cells

1.6.1 Cell agglutination

Agglutination is the most common phenomenon of interaction between lectins and cells. Agglutination of red blood cells or hemagglutination is conventional method to test lectin agglutination activity. In this assay, extracts from plants or animals are serially diluted and a suspension of mammalian erythrocytes, e.g., human, sheep, and rabbit erythrocyte, either enzyme-treated or untreated cells, is added to the solution. The agglutination activity can be assayed macroscopically (Kilpatrick and Yeoman, 1978) or spectrophotometrically (Liener, 1955; Lis and Sharon, 1973; Miller, 1983). Some lectins, for example peanut agglutinin (PNA), cannot agglutinate normal but neuraminidase-treated erythrocytes. It has been suggested that neuraminidase or trypsin treatment of cells exposes surface glyco proteins and glycolipids and hence increases the number of lectin-binding sites on the cells (Lis and Sharon, 1977).

Due to the fact that lectins contain two or more sugar-binding sites, they can cross-link between two adjacent cells and cause cell agglutination. This phenomenon could be reversed by adding sugar specific to those lectins. Lectins show different agglutination between normal and malignant cells, between embryonic and adult cells. Transformed cells are usually found to be agglutinated at a much lower concentration of lectin than is required for the corresponding normal, untransformed cell type (Brown and Hunt, 1978). Change in lectin agglutinatibility has been found to accompany with neoplastic transformation.

Various studies involving lectin-induced agglutination have been carried out on spermatozoa. Lectin agglutinability has been found to change with sperm maturation. Olson and Danzo (1981) reported the agglutination of rat sperm, induced by ConA, changed as the sperm transit from caput to cauda epididymis. Caput epididymal sperm were agglutinated by lower amount of ConA. Agglutinin-120 (RCA) and *Ulex europaeus* agglutinin type I (UEA) have also been found to induce a high degree of agglutination with ram caput sperm, but this capacity was lost as sperm progressed through the epididymis. Wheat germ agglutinin (WGA) did not induce agglutination of sperm from the caput or corpus but did induce agglutination of sperm from the proximal and distal cauda epididymis (Hammerstedt et al., 1982). The observation above suggests the modification of sperm surface in such a way that lectin-binding sites have been changed.

1.6.2 Mitogenic stimulation

When lymphocytes are treated with a mitogenic lectin, they undergo "blast" transformation, they increase progressively in size, endocytosis is stimulated, cytoplasmic vacuoles appear, and many metabolic processes are stimulated including DNA, RNA, and protein synthesis. Many lectins have been reported to be mitogens, for example, PHA-the first known mitogen, ConA, pokeweed mitogen (PWM), lentil lectin, and lima bean lectin. Some lectins stimulate both T and B cells, the others interact with either T or B cells. For instance, ConA and PHA stimulate T cells but not B cells while pokeweed mitogen (PWM) stimulates both types of cells. Table 3 shows lectins known to be mitogens and their cell specificity.

TABLE 3 Mitogenic lectins¹

<u>Lectin</u>	<u>Cell Specificity</u>	
	<u>T</u>	<u>B</u>
<i>Abrus precatorius</i> *		
<i>Agaricus campestris</i> * (wild-meadow mushroom)		
ConA	+	
<i>Geodia cydonium</i> : I* (sponges)		
Lentil	+	
Lima bean		+
<i>Phaseolus vulgaris</i>	+	
Pokeweed mitogen	+	+
<i>Robinia pseudoacacia</i>	+	+
<i>Sophora japonica</i> *		
Soy bean agglutinin	+	
<i>Ulex europaeus</i> I	+	
<i>Vicia faba</i> *		
Wax bean*		
<i>Wistaria floribunda</i>		
mitogen (WFM)*		

¹ modified from Lis and Sharon (1977)

* Cell specificity not known

The mechanism by which lectins exert the stimulating effect is obscure, but it is generally accepted that the initial step in the sequence of events leading to mitogenesis-as to cell agglutination-is binding of the lectin through its sugar binding sites to cell surface receptors (Sharon and Lis, 1975). The removal of the lectins from cell surface by specific sugars blocks the cell stimulation. It is generally believed that the binding of lectin generates a trigger signal at the level of plasma membrane and this transmission of this signal to the interior of cell results in a series of biochemical events (Sharon and Lis, 1975). The earliest changes include a transient increase in adenylyl cyclase-cAMP, increased permeability for glucose, amino acids and ions such as K^+ and Ca^{2+} , acetylation of histone, phosphorylation of nuclear proteins and modification of lipid and carbohydrate metabolism. Later, RNA, protein, and DNA synthesis increase and cells enter mitosis. Apart from these events, the stimulated lymphocytes release soluble factors or lymphokines, for example migration-inhibitory factor (MIF), lymphotoxin and interferon (Lis and Sharon, 1977).

1.6.3 Other effects

Several lectins are highly toxic to mammalian cells and some of these preferentially kill transformed cells. This cytotoxic effect of lectins is, as expected, inhibited by specific sugars. Among all cytotoxic lectins known, ricin (*Ricinus communis* lectin) and abrin (*Abrus precatorius* lectin) are the most toxic substances. Ricin and abrin are composed of two subunits, A and B. For the action of ricin, the lectin would bind to cell surface receptors

through the B subunit. Subsequently the lectin enters into the cell where the A and B subunits dissociate and the A subunit binds to ribosomes to inhibit protein synthesis by catalytic inactivation of ribosomal factors (Olsnes and Pihl, 1974). The mode of action of abrin is similar to ricin. The exact mechanism of other toxic lectins, e.g., ConA, WGA, PHA to exert their activity has not been known, but binding to carbohydrate receptors is an essential first step in each case and one event associated with cytotoxicity may be to inhibit membrane transport of nutrients (Hughes, 1983).

Apart from erythrocytes, lymphocytes, spermatozoa, and transformed cells as mentioned, lectins have been found to bind the other types of cells, such as sea urchin eggs, mammalian eggs, and eggs of other species, blood platelets, liver fat cells and retinal disc membrane (Sharon and Lis, 1975). The binding of lectins to cells mimics the reactions caused by "natural agents". The binding of ConA to platelets result in a "platelet release reaction" (Santoro, 1983) that is similar to normal thrombin - or ATP-induced platelet release reaction (Sharon and Lis, 1975). Lectins can also mimic many insulin actions on the intact cells. These lectins presumably bind to the plasma membrane insulin receptors, generate the same signal(s) as insulin and are internalized (Purrello et al., 1983). Purrello et al (1983) has also found that ConA and PHA stimulate ^{32}P incorporation into the nuclear envelope to the same extent as insulin itself. ConA and PHA, at suitable concentration, inhibit insulin action by competitive binding with insulin receptors on cell membrane.

It has been reported (Nicolson, 1974) that ConA and WGA effectively block the fertilization of sea urchin eggs. Subsequently,

it has been found that effect of WGA is not primarily due to its interaction with the egg surface, but rather with the sperm. On the other hand, blocking of fertilization of mammalian eggs by lectins appears to be the result of the lectin-binding to the outer coat surrounding the egg (zona pellucida) (Nicolson, 1974).

1.7 Vertebrate lectins

Vertebrate lectins can be divided into two groups-the soluble lectins and integral membrane lectins. Integral membrane lectins appear to have evolved to bind glycoconjugates to membrane, either on the cell surface or within vesicles (Barondes, 1984). This results in the localization of the glycoconjugates at particular membrane sites or their transport to other cellular compartment. The soluble lectins, in contrast, move freely in the aqueous compartments within and between cells, interacting with both soluble and membrane-bound glycoconjugates (Barondes, 1984).

Like plant lectins, an important characteristic of the vertebrate lectins is that they have binding specificity for sugars which are commonly found in cell membrane and secretory glycoproteins (Olden et al., 1982). They can agglutinate red blood cells, the property which is common for lectins. A list of vertebrate lectins so far studied is shown in Table 4. In addition to lectins shown in the table, fibronectin and laminin can also bind heparin and agglutinate erythrocytes. Both should have been classified as lectins.

TABLE 4 Vertebrate lectins (from Olden et al., 1982,
and Barondes, 1984)

<u>Lectins</u>	<u>Major source</u>	<u>Subunit</u> <u>MW</u>	<u>Sugar</u> <u>Specificity</u>
I. <u>Integral mem-</u> <u>brane lectins</u>			
1. Gal-binding lectin	Mammalian liver	40,000-55,000	GalNAc>Gal,Glc
2. GalNAc- binding lectin	Avian liver and lower livels in kidney, heart, lung, spleen, testis, duodenum and pan- creas	26,000	GalNAc
3. Man-6-P binding lectin	Mammalian testis, spleen, liver, kidney	215,000	Man-6-P

TABLE 4 (continued)

<u>Lectins</u>	<u>Major source</u>	<u>Subunit MW</u>	<u>Sugar Specificity</u>
II. <u>Soluble lectins</u>			
1. Lactose binding lectin	Many mammalian and avian tissues	15,000-17,000	Gal(β 1-4)- GlcNAc Gal(β 1-4)Glc, Gal(β 1-4)- GalNAc
2. Heparin binding lectin	Rat lung, chicken muscle and liver	13,000-16,000	Heparin, GalNAc
3. Intestinal lactose binding lectin	Secretory granules of intestinal goblet goblet cells	14,000	Gal (β 1-4)- Glc

TABLE 4 (continued)

<u>Lectins</u>	<u>Major source</u>	<u>Subunit MW</u>	<u>Sugar Specificity</u>
4. Calf- β -galac- toside lectin	Heart, lung, spleen, muscle	13,000	β -galactoside
5. Chicken lactose lectin I	Lung	15,000	β -galactoside
6. Rat β -galac toside lectin	Lung	13,500	β -galactoside
7. Human β -galac- toside lectin	Lung	14,000	β -galactoside
8. Thrombolectin	<i>Bothrops atrox</i> (snake) venom	15,000	β -galactoside
9. Chicken lactose lectin II	Intestinal mucosa	14,000	β -galactoside
10. Erythroid developmental agglutinin	Rabbit bone marrow	13,000	β -galactoside
11. <i>Anguilla</i> <i>rostrata</i> (eel) lectin	Serum	10,000	L-fucose
12. <i>Xenopus laevis</i> lectin	Egg, embryo	43,000; 45,000	α -or β -galac- toside

TABLE 4 (continued)

<u>Lectins</u>	<u>Major source</u>	<u>Subunit MW</u>	<u>Sugar Specificity</u>
13. <i>Rana</i> <i>catesbiana</i> lectin	Egg	210,000	β -galactoside
14. <i>Rana japonica</i> lectin	Egg	13,500	Glycoprotein rich in sialic acid

1.8 Biological roles of lectins

1.8.1 Plant lectins

As mentioned above, lectins are very diverse, they vary in molecular structure and sugar specificity. Lectins also come from different sources or different part of plants. Thus, the biological roles of lectin may vary. Some proposed biological roles of plant lectins is outlined below.

1.8.1.1 Binding nitrogen-fixing bacteria to legume roots

The majority of studies on the biological role of lectins involve the interaction between Leguminosae lectins and soil bacteria of the genus *Rhizobium* (Reviewed in Barondes, 1981). These bacteria fix nitrogen and provide nitrogen source required for plants. The bacteria adhere to the root and become internalized to form nitrogen-fixing nodules. It has been found that the majority of *Rhizobium* strains studied bind specifically with the lectin of their respective host plants. Apparently, the bacterial exopolysaccharide serves as a target for the lectin (Rudiger, 1984). It has been postulated that lectin may be bound to the outer root surface, thereby fixing bacteria from the surrounding soil (Rudiger, 1984). The other hypothesis is that bacteria and root surface share a common site which binds specifically with lectin and the lectin acts as a glue between root and rhizobia (Rudiger, 1984). Evidence for lectin involvement in binding of *Rhizobium* to soy bean root hair was first provided by Bohloul and Schmidt (1976). Soy bean lectin from some, but not all, nodulating soy bean plants have been found. The failure of some nodulating strains to exhibit the activity of soy bean lectin seems

puzzling. Following the first study by Bohlool and Schmidt (1976), many studies have been reported either for or against the hypothesis. The "lectinless" strain of soybean cannot be nodulated by *Rhizobium*. However, plant strains regarded as being devoid of lectin may contain a different lectin or lectin like substance (Pusztai et al., 1981). This has been confirmed by Dombrink-Kurtzman et al (1983) that soy bean seeds contain a new lectin activity which has specificity towards the 4-O-methyl-D-glucurono-L-rhamnan exopolysaccharide produced by certain strains of *Rhizobium japonicum*. Even the "lectinless" strains contain this lectins. However, a final proof of this proposed role of lectin requires much further study.

1.8.1.2 Protection against pathogens

Wheat germ agglutinin (WGA), which has the specificity towards chitin oligomer, has been found to inhibit chitin synthesis, hyphal growth, and spore germination of *Trichoderma viride* and *Fusarium solani* (McDonough and Lilien, 1975). It is possible that WGA protects wheat against chitin-containing phytopathogen during seed germination and early seedling growth (McDonough and Lilien, 1975). Lectins with sugar specificities different from that of WGA may inhibit the growth of other soil microorganisms. The lectin from common potato enables germinating *Phytophthora* spores to bind to potato cell membranes and to agglutinate the pathogenic cells. These effects can be inhibited by oligosaccharides produced by the degradation of chitin, which are specific for this lectin (Rudiger, 1984).

1.8.2 Lectins in slime molds

The slime mold, *Dictyostelium discoideum*, contains two

lectins, discoidin I and discoidin II. Each is a tetrameric protein with molecular weight of approximately 100,000. Both bind β -galactosides, but discoidin I has a much higher affinity for N-acetylgalactosamine (Barondes, 1984). Discoidin I level becomes maximal as aggregation is completed, and declines thereafter while discoidin II level becomes maximal during fruiting body formation. Early in aggregation, discoidin I is mostly intracellular in large clusters. The lectin is externalized with further differentiation, and become associated with the slime coat (Barondes, 1984). Due to the fact that discoidin I is found on slime surface and that the synthesis of discoidin I progresses as cell-cell adhesion develops, its possible role in this process has been considered. Lectins may bind to the glycoconjugates and form the cross-bridge between the two opposing cells, leading to cell adhesion.

1.8.3 Lectins in vertebrates

The presence of integral membrane lectins has been considered to involve in transport of the glycoproteins. Galactose-binding lectin, for example, on the membrane of liver cells has been suggested to mediate clearance of partially degraded glycoproteins (Stockert et al., 1980). This lectin may interact with terminal galactose residue of the glycoproteins. After binding, the lectin-glycoprotein complexes are internalized *via* endocytosis. The complexes are transported to lysosome where they will be degraded. Several other vertebrate lectins have been identified and these also appear to function in cellular translocation of glycoproteins (Barondes, 1984).

A common property of soluble vertebrate lectins is that

they are maximally synthesized at specific stages in the embryonic development of certain tissues. For example, chick lactose lectin I (CLL-I) level in embryonic muscle increases as the tissue differentiates (Nowak et al., 1976) and decline thereafter. CCL-II levels in liver and kidney are much higher in the embryo than in the adult (Barondes, 1981). It has been found that soluble vertebrate lectins appear to be secreted. In some cases, there is a shift from an intracellular to an extracellular location during differentiation. CLL-I is concentrated intracellularly in developing chicken muscle but becomes extracellular during maturation (Barondes and Haywood Reid, 1981). The secretion of CLL-I is accompanied by polynucleated myotube formation, suggesting that lectins participate in cell fusion.

1.9 Applications of lectins

1.9.1 Fractionation of membrane and other glycoproteins

Since lectins recognize and bind specifically to sugar sequences of glycoprotein, they can be used in fractionation of membrane-bound and soluble glycoproteins. Material is passed through the immobilized lectin and the adsorbed glycoprotein is eluted with sugar for which the lectin is specific.

For membrane bound glycoproteins, they should be first solubilized from the membrane by using a detergent, for example 0.5% Triton X-100. Then the proteins are adsorbed to immobilized lectin and subsequently eluted with appropriate sugars.

By this technique, many soluble glycoproteins and membrane bound glycoproteins have been fractionated. For example, blood group A substance from ovarian cyst fluid has been fractionated on *Vicia*

cracca PHA-Sepharose (Kristiansen, 1974). *Lotus* and *Dolichos* lectins have been proven very satisfactory for the isolation from hog gastric mucin of fractions showing only A, only H, and a small amount of both activities (Pereira and Kabat, 1976). Lymphocyte membrane glycoproteins were purified on ConA-Sepharose and on Lentil-Sepharose (Kornfeld and Kornfeld, 1978). Peterson et al (1981) fractionated plasma membrane proteins from boar spermatozoa by using an ion exchange chromatography and WGA agarose. Barber and Arya (1982) fractionated and identified pig lymph node plasma membrane glycoproteins. They solubilized the plasma membrane with 1% (w/v) sodium deoxycholate and fractionated the soluble fraction by gel filtration and by lentil lectin-Sepharose.

Immobilized lectins also have potential for fractionating cell populations. For example, when mouse spleen cells were passed through a ConA-polyacrylamide column, the cells which appeared in the effluent bound 2.4-fold less ConA than the applied cell population while the cells which were bound to the column and were eluted with methyl- α -D-mannoside had a 5.2-fold enrichment in ConA binding (Kornfeld and Kornfeld, 1978).

Lectins have also been used in separation of mouse T and B spleen lymphocytes (Lis and Sharon, 1977). The B cells were agglutinated by soy bean agglutinin (SBA); the agglutinated cells were separated by sedimentation and were then dissociated by galactose.

1.9.2 Study of cell surface

Since lectins can bind the cell surface glycoproteins, they can be used as probes to explore the number and distribution of receptor molecules on cell membrane. Lectins are first labelled

by fluorescent dyes, ferritin, or radioactive agents, and allowed to bind to surface of cells. Lectin binding can be observed by detecting the presence of labelled lectins on the cell membrane. For example, ferritin-conjugated *Phaseolus vulgaris* lectin can bind to intact erythrocytes or erythrocyte membranes and then the bound lectin can be visualized using electron microscopy. Tillack et al (1972) have shown that the *Phaseolus vulgaris* PHA receptors are distributed uniformly on red cell membrane. Major sialoglycoproteins of red cell membrane have been shown to be the receptors for peanut lectin (Carter and Sharon, 1977).

Lectins have also been used in studying sperm surface and surface change during sperm maturation. The first group, Kashiwahara et al (1965) have found that soy bean agglutinin (SBA) from a variety of strains interacts with bull spermatozoa to produce predominantly tail-to-tail agglutination. Badel and Brilliantine (see Koehler, 1983) have utilized a vast number of plant and seed extracts to study agglutination of sperm from several species of clam. Instead of tail-to-tail agglutination, however, only head-to-head interaction have been observed. Nicolson and Yanagimachi (1972) have used six lectins with different sugar specificity to study rabbit and hamster spermatozoa. Some species difference have been detected. For example, *Dolichos biflorus* agglutinin (DBA) does not agglutinate hamster sperm suggesting that N-acetyl-D-galactosamine is not a significant terminal saccharide in this species whereas rabbit sperm was strongly agglutinated. The pattern of agglutination, tail-to-tail, head-to-head, or head-to-tail, depends on the localization of lectins. Tail-to-tail agglutination means that the lectin receptors are mostly

localized on the membrane of sperm tail. However, the exact localization of receptor can be shown by using labeled lectins. For example, ConA-binding receptors on rat sperm membrane have been shown, by using fluorescein isothiocyanate-conjugated ConA, to be on the entire sperm surface of caput sperm. However, in cauda sperm these receptors are mostly on the acrosome (Olson and Danzo, 1981).

Lectins have been used to probe the changes in nature of the sperm surface during maturation. For rabbit sperm, WGA and RCA are bound progressively less to sperm as they proceed from caput to cauda epididymis and to the ejaculate whereas ConA binding shows very little alteration (Koehler, 1983). The major changes during sperm maturation involve the loss of receptors on the sperm except in the case of ConA. Hammerstedt et al (1982) have studied in ram sperm and found that RCA and UEA I induce a high degree of agglutination with caput sperm, but this capacity is lost as sperm progress through the epididymis. In contrast, WGA does not induce agglutination of sperm from caput or corpus but induce agglutination of sperm from the proximal and distal cauda epididymis. A more recent study of Fain-Maurel et al (1983) shows that the binding of ConA to sperm of *Macaca fascicularis* (monkey) is observed in distal corpus, and increases in cauda epididymis. Wheat germ agglutinin (WGA), however, uniformly binds to plasma membrane of the head of the sperm from caput epididymis, the reaction becomes negative during the sperm passage through corpus and cauda epididymis.

Taken together these studies indicate that binding sites for a number of lectins either increase or decrease during the progressive passage of sperm from the testis to the caudal portion of

epididymis. It may be due to alterations of surface molecules by proteases or glycosidases, addition of new sugar residues by glycosyltransferase or adsorption of masking substance (Fain-Maurel et al., 1983).

1.9.3 Identification of bacteria and fungi

Lectins have been known to bind selectively to microorganisms. The binding of lectins to cell surface of microorganisms is similar to that of erythrocytes in that lectins bind specifically to carbohydrate residues or sequences of molecules present on cell surface. The lectin receptors on red blood cells are usually glycoproteins, but in the case of bacteria and fungi, lectin receptors are polymer of simple sugars, e.g., mannan, chitin, teichoic acid, and lipopolysaccharide (reviewed by Pistole, 1981). Other microbial polysaccharides that interact with lectins are dextrans, levans, glycogens and heteropolymeric mannans.

Among lectins known, ConA has been widely used in study of lectin-binding to microorganisms. For example, Hamada et al (1977) reported that 13 of 15 strains of *Streptococcus mutans* tested were agglutinated by ConA. Other organisms agglutinated by ConA are *Streptococcus sanguis*, *Salmonella* spp., *Mycoplasma* spp., *Staphylococcus aureus*, *Saccharomyces cerevisiae* (baker's yeast), *Candida* spp., *Fusarium* spp., *Aspergillus* spp., *Penicillium* spp., and others (Pistole, 1981; Doyle and Keller, 1984). It seems likely that ConA, which has the specificity towards α -D-glucose and α -D-mannose, interacts with α -D-glucosylated teichoic acids of gram-positive bacteria. With gram-negative bacteria, ConA bind to exposed sugars on the lipopoly-

saccharide (LPS). *Neisseria gonorrhoeae* are agglutinated by WGA, presumably via LPS on the cell surface (Frasch, 1980). *Neisseria meningitidis* and *Neisseria lactamica* are also agglutinated by this lectin (Doyle et al., 1984). Other species of *Neisseria*, and *Branhamella catarrhalis*, are not agglutinated by WGA. Lectins from *Ricinus communis* (RCA), soy bean (SBA) and *Griffonia simplicifolia* also agglutinate *Neisseria gonorrhoeae* (Doyle et al., 1984).

Since lectins can bind selectively with varieties of microbial structures, and different microorganisms have different exposed carbohydrate structures, it is of interest to use lectins to identify those microorganism. For example, in genus *Neisseria*, as mentioned previously, different species of bacteria show different pattern of lectin-induced agglutination. Several studies have described the use of WGA for distinguishing between members of the *Neisseriaceae*. Recently, Yajko et al (1984) have utilized WGA, SBA and chromogenic substrates, e.g., γ -glutamyl- β -naphthylamide, as a rapid method for identification of *Neisseria gonorrhoea*. Doyle et al (1984) have also used WGA and γ -glutamyl- β -naphthylamide. WGA agglutinates both *Neisseria gonorrhoea* and *Neisseria meningitidis*. *Neisseria meningitidis* produces enzyme γ -glutamyl aminopeptidase that hydrolyzes γ -glutamyl- β -naphthylamide while *N. gonorrhoea* does not produce that enzyme. Thus, WGA test coupled with the assay for γ -glutamyl aminopeptidase offers a convenient, rapid and accurate means of identifying *N. gonorrhoea* from primary isolation media.

Sliekin and Gil (1984) have reported the method to identify group C Streptococcal antigen using *Dolichos biflorus* lectin coupled

to polystyrene particles. Group C Streptococcal antigen extracts were specifically agglutinated by this lectin-bound matrix. This serves as a definitive identification of group C Streptococci. Pathogenic fungi have also been detected and identified in parafilm sections of surgical and postmortem specimen by using lectins (Stoddart, 1978). Both hyphae and septa of *Aspergillus fumigatus* and *Rhizopus oryzae* were highly reactive with SBA and WGA.

1.9.4 Selection of cell lines with altered membrane carbohydrate

Since certain lectins, for example ConA, WGA, PHA, are cytotoxic, addition of these lectins to cell culture may select for lectin-resistant variants. The surviving lectin-resistant cells are then cloned and tested for altered membrane property.

The earliest attempt at obtaining cultured cell mutants which fail to interact with specific lectins were made by Wright (1973) who isolated chinese hamster ovary (CHO) cells selected for resistance to ConA and PHA. Gottlieb et al (1974) used ricin to select a clone of CHO cells which were deficient in membrane-binding sites for a number of lectins. The membranes of this cell line were shown to have decreased amounts of sialic acid; galactose, and N-acetylglucosamine compared to the membranes of the parent cells. Ricin-resistant variants of CHO cell, with more sialic acid attached to lectin receptors, were selected by Stanley and Siminovitch (see Briles, 1982). Other variants with deletion of oligosaccharides, for example WGA-resistant variants, PHA-resistant variants and pea lectin-resistant variants have also been selected (reviewed by Briles, 1982).

The availability of lectin-resistant cell lines with a

variety of alterations in membrane structure and function provides and opportunity to study various aspects of the structure, biosynthesis and function of cell membrane glycoproteins and oligosaccharide units (Kornfeld and Kornfeld, 1978).



1.10 Aims of Thesis

1. To survey plants in Thailand for new lectin sources.
2. To purify and characterize Jack fruit lectin.



2. MATERIALS AND METHODS

2.1 Chemicals

Sodium chloride was purchased from Famitalia Carlo Erba. Sodium hydroxide, Sodium phosphate, mono- and di- basic were from Riedel De Haeng Seelze-Hannover. Sodium potassium tartrate, copper sulfate, and sodium carbonate were products of J.T. Baker Chemical Co. Folin Ciocalteu "Phenol reagent" was obtained from Fisher Scientific Company.

Ammonium sulfate and ethanolamine were supplied by MERCK. Antisera to lectins were from Sigma Chemical Co. Wheat germ agglutinin (WGA), peanut agglutinin (PNA) and Concanavalin A (ConA) were obtained from Bethesda Research Laboratories Inc. *Phaseolus vulgaris* phytohemagglutinin (PHA) was from Sigma Chemical Co. Neuraminidase (*Vibrio cholera*) was obtained from Calbiochem. The seeds of Thai fruits were purchased from local suppliers.

Ficoll 400 and Sephadex G-200 were from Pharmacia Fine Chemicals. Epoxy-activated Sepharose 6B was obtained from Sigma Chemical Co. Affigel-galactosamine was from Biorad. Acrylamide, SDS, glycine, EDTA, β -mercaptoethanol, coomassie Brilliant blue R-250, Tris and all molecular weight marker proteins were also from Sigma. N,N' -methylene bisacrylamide, TEMED, and urea (ultra pure) were purchased from Eastman Kodak Co.

2.2 Experimental animals

Male albino rats (Fischer strain) were supplied by The Animal Center, Faculty of Science, Mahidol University, Bangkok, Thailand.

The rats were 2-3 months of age and weighed 250-300 g.

2.3 Preparation of rat sperm

The rats were anaesthetized with ether and the epididymidis were removed. Each epididymis was dissected free of fat and cut at mid corpus region to give two pieces of tissues: caput and cauda. After washing with 5 mM phosphate buffer saline (PBS) pH 7.4, the caput and cauda epididymides were punctured in PBS using a hypodermic needle. The sperm count was determined using a hemocytometer and adjusted with PBS to 4×10^7 cells/ml.

2.4 Human sperm

Semen samples were collected by masturbation into clean bottles and were delivered to the laboratory within 2-3 h. Samples were examined for the sperm count and bacterial contamination. Semen samples contaminated with bacteria were discarded. Semen was diluted with PBS until the concentration of sperm was 4×10^7 cells/ml.

To wash human sperm, semen was overlaid on a layer of 10% Ficoll (in PBS) and centrifuged at $500 \times g$ for 30 min. After centrifugation, the supernatant was discarded and the sperm pellet was resuspended in PBS. The concentration of sperm was adjusted to 4×10^7 cells/ml with PBS.

2.5 Preparation of human erythrocytes for hemagglutination test

Human blood was obtained fresh from the volunteers by venipuncture. The blood was mixed with the anticoagulant, acid citrate dextrose (ACD). The plasma was removed after centrifugation at $500 \times g$ for 5 min. Red blood cells were washed three times with PBS and then a 2% suspension of cells was prepared.

2.6 Neuraminidase treatment of red blood cells

An aliquot (0.05 ml) of neuraminidase (1 IU/ml) was incubated with 2.5 ml of 2% red cell suspension at 37°C for 1 h. After incubation, the cells were washed three times with PBS and a 2% suspension of the enzyme-treated cells was prepared in PBS.

2.7 Extraction of lectin from seeds

The seeds were homogenized in PBS using Waring Blender at full speed for 3 min, stirred at 4°C for 2 h and then centrifuged at $3,000 \times g$ for 15 min to remove insoluble debris. Ammonium sulfate was added to the extract up to 80% saturation with constant stirring. The precipitate which formed during 15-20 min stirring was collected by centrifugation at $27,000 \times g$ for 15 min, resuspended in PBS and dialyzed for 24 h with repeated change of buffer. Any precipitate formed during dialysis was later removed by centrifugation at $27,000 \times g$ for 15 min. The final clear supernatant was collected and kept at -20°C .

2.8 Protein determination

Protein concentration was determined by the method of Lowry et al (1951). Bovine serum albumin was used as standard.

2.9 Agglutination tests

2.9.1 Rat sperm agglutination test

An aliquot (10 μ l) of rat sperm suspension (4×10^7 cells/ml) was mixed with 50 μ l of seed extracts at room temperature. A drop of the mixture was immediately transferred onto a microscopic glass slide. The clumping of sperm was observed within 1 min after the mixing under dark-field microscope. Sperm agglutination was scored as 0, 1, 2, 3, 4 according to approximate size of the aggregate (see Fig. 2).

- 0 : no sperm agglutination, all sperm were free
- 1 : small clump, the number of sperm/clump was 5-10
- 2 : moderate clump (11-25 sperm/clump)
- 3 : big clump (25-35 sperm/clump)
- 4 : massive clump (>36 sperm/clump)

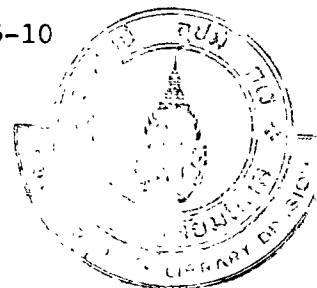
The sperm were also incubated with PBS as the negative control.

2.9.2 Human sperm agglutination test

An aliquot (50 μ l) of unwashed or washed human sperm suspension (4×10^7 cells/ml) was incubated with 50 μ l of seed extracts at room temperature. Sperm agglutination was observed and scored within 1 min after the mixing as in rat sperm agglutination (see Fig.2)

2.9.3 Hemagglutination test

Seed extracts (50 μ l) were incubated with an equal volume of 2% red blood cells, untreated or neuraminidase-treated, in test tubes at room temperature for 1 h. Hemagglutination was observed macroscopically and scored as 0, 1, 2, 3, 4 according to the size of



hemagglutination (see Fig. 3).

2.9.4 Agglutination activity

Agglutination activity of a seed extract was determined according to the method of Kilpatrick and Yeoman (1978). Seed extracts were serially diluted (2-fold dilution) and these diluted fractions were incubated with human erythrocytes, human sperm or rat sperm as above (2.9.1-2.9.3). The titer was the highest dilution that caused agglutination at the score 1. Activity was taken to be the reciprocal of the titer. For example, if the titer is 1/16, the activity would be 16 units/volume of seed extracts used. The specific activity was the activity per milligram of protein.

2.10 Agglutination inhibition test

2.10.1 Hemagglutination inhibition test

A seed extract giving positive hemagglutination test in section 2.9.3 was diluted with PBS to the concentration that caused hemagglutination at the score 2. The test was repeated using the diluted extract in the presence of 25 μ l of a test sugar (in PBS) or in the presence of 25 μ l of PBS (control). The minimal concentration of sugar that fully inhibited hemagglutination was recorded.

2.10.2 Sperm agglutination inhibition test

A seed extract giving score 4 in sperm agglutination tests (section 2.9.1-2.9.2) was diluted with PBS to the lowest concentration that still gave a score of 4. The test was repeated using such lowest concentration in the presence of 25 μ l of a test sugar (in PBS) or in the presence of PBS (control). The minimal concentration of sugar that

completely inhibited sperm agglutination was recorded.

2.11 Heat stability test

Seed extracts were incubated at 80°C for 15 min. Any precipitate formed was removed by centrifugation at 27,000 x g for 15 min at 4°C. The clear supernatant was collected and tested for sperm agglutination activity and compared with that of an unheated aliquot.

2.12 Initiation of sperm motility

Rat cauda epididymal sperm freshly removed from the tubule without dilution are non-motile. The motility can be initiated by diluting with a salt solution such as PBS. The sperm motility initiation was performed as described by Chulavatnatol (1982).

Seed extracts in PBS were tested for their ability to inhibit the initiation of rat cauda epididymal sperm motility. Cauda epididymidis were surgically removed from the rat and placed in watch glasses containing paraffin oil. An aliquot (0.1-1 µl) of the epididymal content containing sperm was extruded from the tubules by puncturing with a hypodermic needle. The extruded mass was pipetted using a pasteur pipet and placed under the paraffin oil on a glass slide. Seed extract (10 µl) was dropped onto the sperm. The motility initiated was observed within 1 min under dark-field microscope and scored as 0, 1, 2, 3, 4. Score of 0 represented no motility being initiated while 4 represented vigorous motility. PBS was used instead of seed extract to give full motility initiation (score 4) and water to give no motility initiation (score 0).

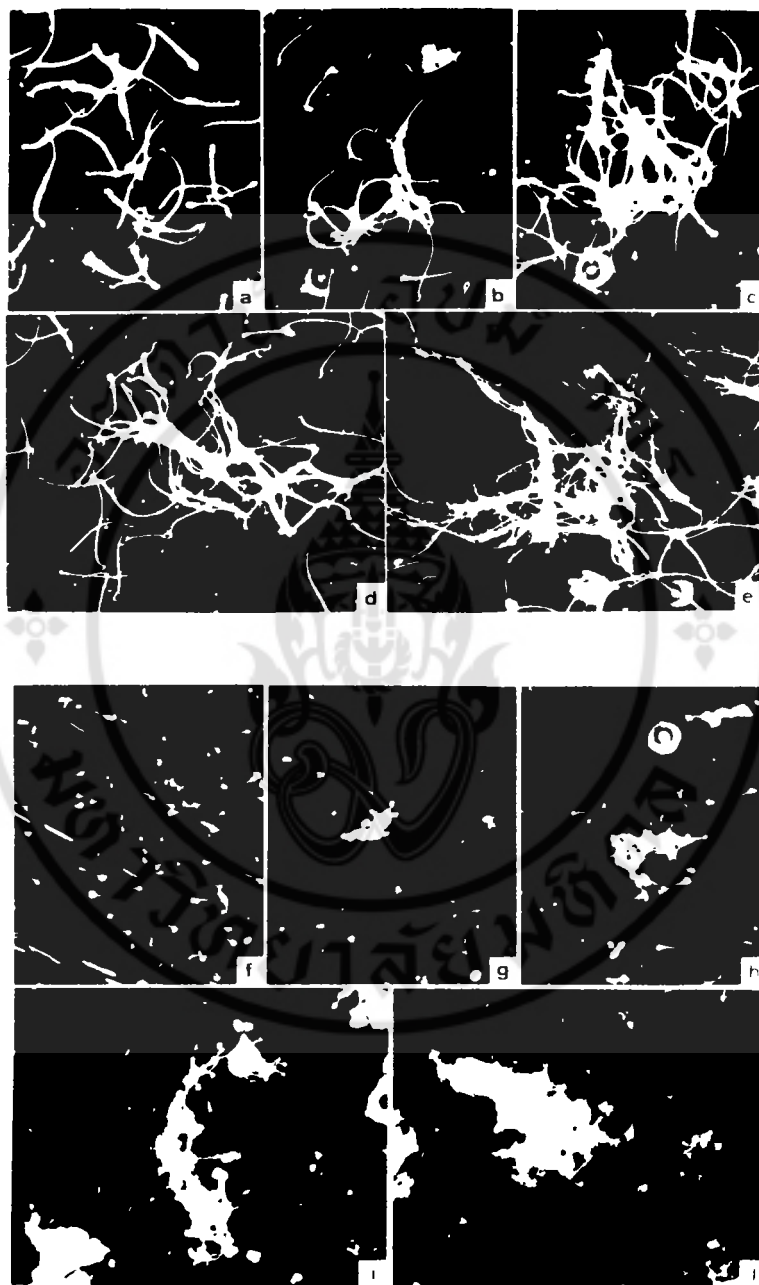


Fig. 2 Scoring of rat sperm (a-e) and human sperm (f-j) agglutination.

a,f = 0; b,g = 1; c,h = 2; d,i = 3; e,j = 4.



Fig. 3 Scoring of hemagglutination : a = 0, b = 1, c = 2
d = 3, e = 4. Arrowhead in (b) indicates
hemagglutination.

2.13 Coupling of GalNAc to epoxy-activated Sepharose 6E

GalNAc was coupled to epoxy-activated Sepharose 6E according to Miller (1983). One gram of epoxy-activated Sepharose 6E was allowed to swell in 100 ml of water. The water was removed by filtration through a sintered glass funnel and the Sepharose was further washed with 400 ml of water. A solution of 6.6 ml of 0.1 N NaOH containing 0.24 g of GalNAc and the moist Sepharose was placed in a tube and mixed by end-over-end tumbling at room temperature for 16 h. The gel was sequentially washed with 100 ml of 0.5 M NaCl followed by 100 ml of PBS. The GalNAc-Sepharose was packed into a column (1.6 x 1.6 cm) and equilibrated with PBS.

2.14 Purification of jack fruit lectin

2.14.1 Chromatography on a GalNAc-Sepharose column

Preliminary study showed that a protein fraction (S-60) precipitated from jack fruit seed extract between 50-60% ammonium sulfate contained most protein and highest agglutination activity (see Fig. 7). An aliquot (50 ml) of S-60 fraction was applied to the GalNAc-Sepharose column at flow rate 10 ml/h. The column was washed with PBS until unbound proteins were completely removed and then 0.1 M GalNAc (or Gal) in PBS was applied to elute the lectin. The sugar-eluted fractions were combined and dialyzed against PBS.

2.14.2 Chromatography on an Affigel-galactosamine column

Affigel-galactosamine consisting of the cross-linked agarose commercially coupled with GalNAc was washed with PBS and packed into a column (1.3 x 4.3 cm). The gel was equilibrated with PBS.

An aliquot of S-60 fraction (50 ml) was pumped into the column at flow rate 10 ml/h. The unbound proteins were completely washed out with PBS. Jack fruit lectin was recovered by eluting with 0.03 M galactose.

2.15 Sephadex G-200 column chromatography

Jack fruit lectin (10 mg in 2 ml PBS) purified from Affigel galactosamine column was applied to a Sephadex G-200 column (1.6 x 90 cm) which was pre-equilibrated with PBS. The protein was eluted from the column with PBS. Protein in a fraction was measured by its absorption at 280 nm and the agglutination activity was determined as described in section 2.9. The column was later calibrated using the following molecular weight markers: blue dextran (MW 2,000,000), ferritin (MW 440,000), aldolase (MW 158,000), ovalbumin (MW 43,000), ribonuclease A (MW 13,700), and DNP-lysine (MW 312).

2.16 Analytical electrophoresis

2.16.1 Non-denaturing electrophoresis (ND-PAGE)

Non-denaturing gel electrophoresis system was modified from Cameo and Blaquier (1976). Sample were treated with 2.5 mM Tris-glycine pH 8.3 and applied to slab gel (15 x 10 x 0.1 cm) with a linear gradient of 5-7% acrylamide consisting of 5-7% (w/v) acrylamide, 0.2% N,N'-methylene bisacrylamide, 0.01 g/ml ammonium persulfate and 0.05% TEMED. The stacking gel (15 x 3 x 0.1 cm) consisted of 3% acrylamide in 0.125 M Tris-HCl pH 6.8. The gels were electrophoresed at room temperature with a constant current of 15 mA.

2.16.2 Sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE)

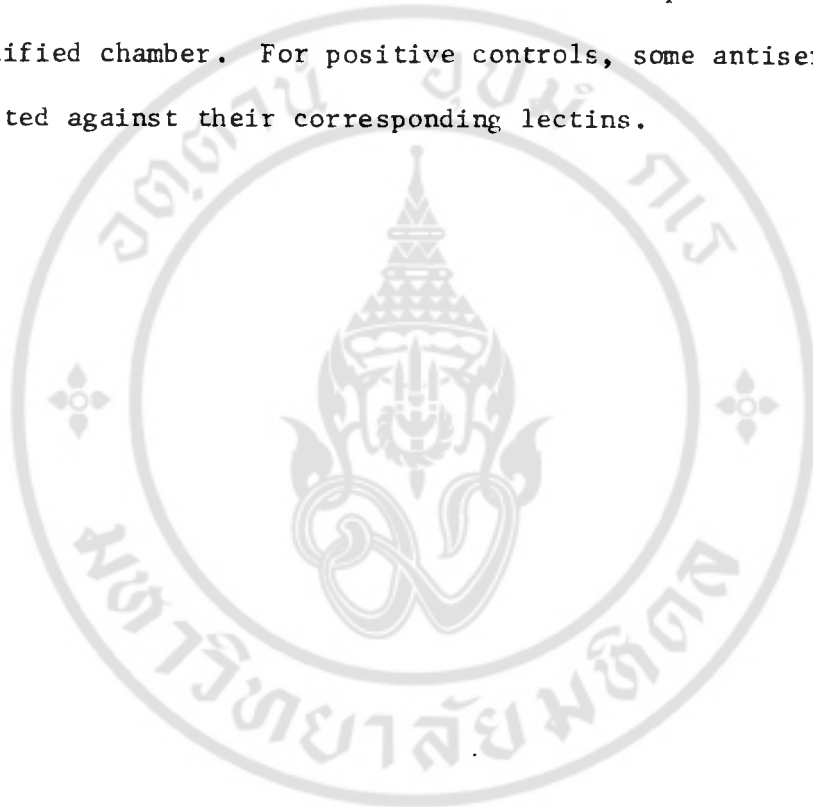
SDS-polyacrylamide gel electrophoresis was carried out by the method of Anderson (1983) which was suitable for low molecular weight proteins. Sample was diluted with sample buffer containing 0.14 M Tris-acetate pH 7.8, 0.5% SDS, and 20% sucrose to the concentration of 2.5 mg protein/ml. An aliquot of SDS-reducing solution (equalling to 1/5 of the diluted sample volume) containing 25 mM DTT, 10% SDS, and 20% sucrose was then added. The treated sample was loaded into a slot of the gel (15 x 10 x 0.1 cm). The separating gel consisted of 8 M urea, 0.2 M Tris-SO₄ pH 7.8, 0.025% SDS, 8% (w/v) acrylamide, 0.025% ammonium persulfate, and 0.05% TEMED. The stacking gel (15 x 4 x 0.1 cm) consisted of 3.125% (w/v) acrylamide, 0.2 M Tris-SO₄ pH 7.8, 0.025% SDS, 0.025% ammonium persulfate and 0.05% TEMED. The electrophoresis was performed at room temperature at constant voltage of 150 V. The lower buffer was 0.2 M Tris-SO₄ pH 7.8 containing 0.04% SDS and the upper buffer was 0.074 M Tris-HCl pH 7.8 containing 0.1% SDS.

2.17 Pronase digestion of jack fruit lectin

Jack fruit lectin (0.2 mg/ml) and S-60 fraction (0.2 mg/ml) were separately incubated with 0.2 mg/ml pronase in PBS pH 7.4 at 37°C for 16 h. After the incubation, the mixtures were diluted 10-fold with PBS. The aliquots from diluted mixtures were tested for agglutination activity.

2.18 Double immunodiffusion

Double immunodiffusion was performed according to William and Chase (1967). Commercial antisera to lectins (5 μ l) were reacted with 5 μ l (1 μ g protein) of jack fruit lectin in 1% noble agar (well separation 0.85 mm, center to center) at room temperature for 24 h in humidified chamber. For positive controls, some antisera were also tested against their corresponding lectins.



3. RESULTS

3.1 Survey of Thai plants for agglutinating agents (lectins)

The seeds of Thai plants were considered as unexplored sources of possible new lectins. Among thirty-one kinds of seeds, one kind of storage root, and one kind of stem tested, thirteen kinds of seeds showed sperm agglutination activity (Table 5). Seed extracts from Jack fruit, red kidney bean, broad bean, garden pea, Sa-taw, green gram, peanut, soybean, and sorghum (dotted) agglutinated both rat sperm and human sperm. In contrast, seed extracts from tamarind, rambutan, and longan agglutinated only rat sperm. For rat sperm, highly active seed extracts showed no difference in sperm agglutination between caput and cauda epididymal sperm (Fig. 4). Extracts from rambutan seeds (Fig. 4, i and j) showed tendency to agglutinate caput epididymal sperm more than cauda epididymal sperm. Longan seed extract was active only with caput epididymal sperm (Table 5). There was no difference between agglutination of unwashed and that of washed human sperm by active seed extracts (Table 5). It seemed likely that Jack fruit extract also caused the agglutination of other materials in seminal plasma (Fig. 5,b).

Seed extract from Jack fruit and green gram appeared to contain at least two active agents, one was heat labile and the other was heat stable (Table 5). Sperm agglutination agents in extract from red kidney bean and longan were heat stable. For other active agents, sperm agglutinating agents were heat labile.

Table 6 shows some seed extracts which were active to both rat sperm and human sperm. The concentration of seed extracts were adjusted to 0.4 mg/ml and 0.25 mg/ml for rat sperm and human sperm respectively, in order to compare their effectiveness. Jack fruit and red kidney bean extracts seemed to contain the most potent sperm-agglutinating agents. Extracts from broad bean and garden pea were somewhat less effective. Seed extract of Sa-taw was very effective with rat sperm, especially caput epididymal sperm, but not very effective with unwashed human sperm. Yard-long bean, green gram, soybean, and peanut contained sperm agglutinating agents that were less active in comparison to those mention earlier. This was confirmed by the results shown in Table 7. In this table, the minimal concentrations needed to achieve agglutination at the score 1 were compared among the active extracts. Jack fruit and red kidney bean extracts were again found to be most effective overall since their effective concentrations were lower than those of other extracts.

Table 5 Sperm agglutination test I. Each seed extract was separately incubated with rat cauda epididymal sperm (RCd), rat caput epididymal sperm (RCp), unwashed human sperm (UHS), or washed human sperm (WHS) and the agglutination was scored as described in section 2.9. Mean $\pm$ SE for n = 4 was then calculated.

For heat stability test, the active seed extract was incubated at 80°C for 15 min. The pellet was removed by centrifugation and the clear supernatant was tested for sperm agglutination (see details in section 2.11).

Table 5

<u>Seed extracts</u>	<u>Protein</u> (mg/ml)	<u>RCd</u>	<u>RCp</u>	<u>UHS</u>	<u>WHS</u>
1. Jack fruit	2.5	4 [*]	4 [*]	4	4
2. Red kidney bean	2.0	4 [*]	4 [*]	4 [*]	4 [*]
3. Broad bean	1.1	4	4	4	4
4. Garden pea	0.5	3.7±0.2	4	2.6±0.8	3.6±0.3
5. Tamarind ^{**}	0.06	3.0±0.5	4	0	0.3±0.3
6. Sa-taw	0.27	2.5±0.5	4	2.0±0.5	2.0±0.5
7. Green gram	3.7	1.2±0.4	3.7±0.2	2.6±0.8 [*]	3.3±0.6 [*]
8. Rambutan	1.0	0.6±0.3	3.6±0.3	0	0
9. Peanut	0.3	1.2±0.2	3.0±0.4	0.5±0.5	3.0±1.0
10. Yard-long bean	0.16	2.0±0.7	2.7±0.6	0	1.6±0.3
11. Soybean	1.0	1.2±0.2	2.7±0.4	0.3±0.3	1.6±0.3
12. Longan	0.24	0	2.7±0.4 [*]	0	0
13. Sorghum, dotted	0.03	1.7±0.6	1.2±0.4	1.5±0.5	1.5±0.5

* heat stabel

** heat stability not tested

the other seed extracts were heat labile

Table 5 (continued)

Seed extracts causing no sperm agglutination



Balsam pear	Winged bean
Black gram	Lotus
Cassava, root	Papaya
Corn	Potato, stem
Castard apple	Rice
Durian	Salacca
Horse-tamarind	Sapodilla
Jamaica sorrel	Sorghum (red)
Job's tear	Sunflower
Litchi	Winged bean

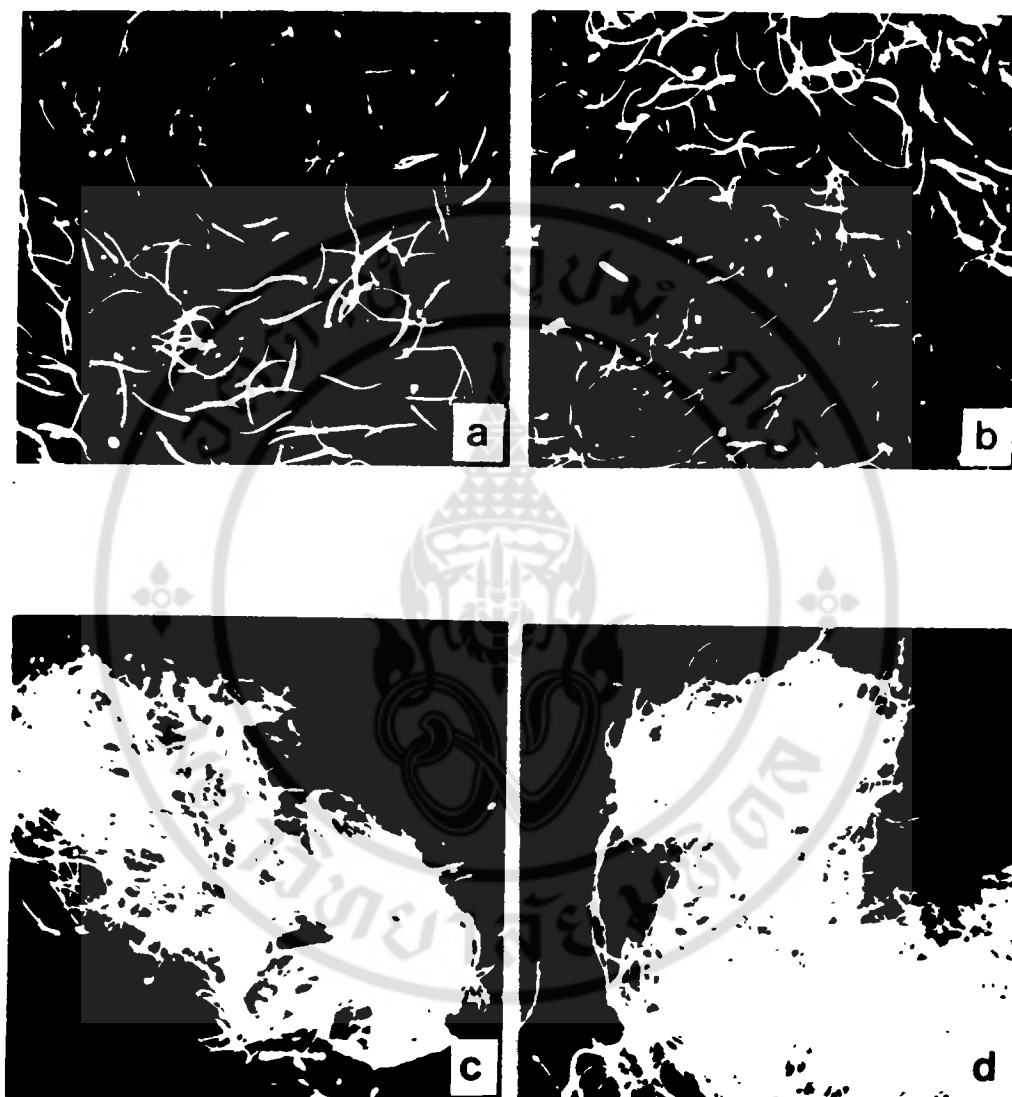
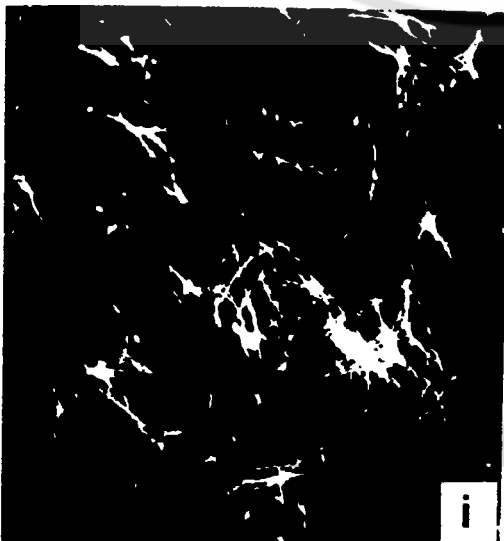
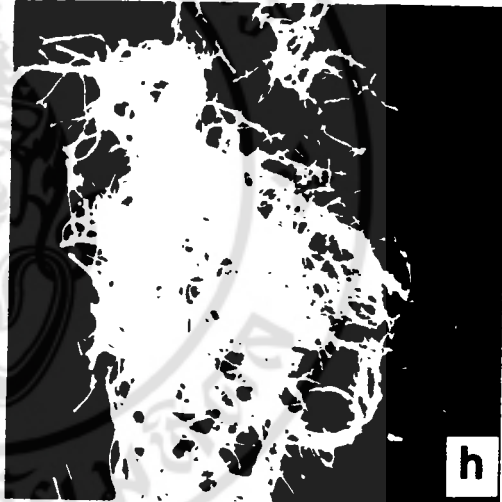
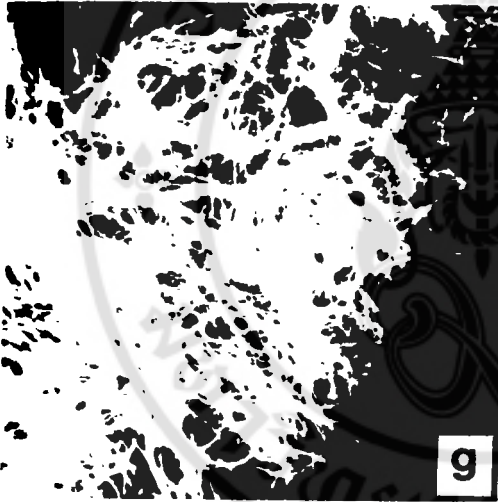
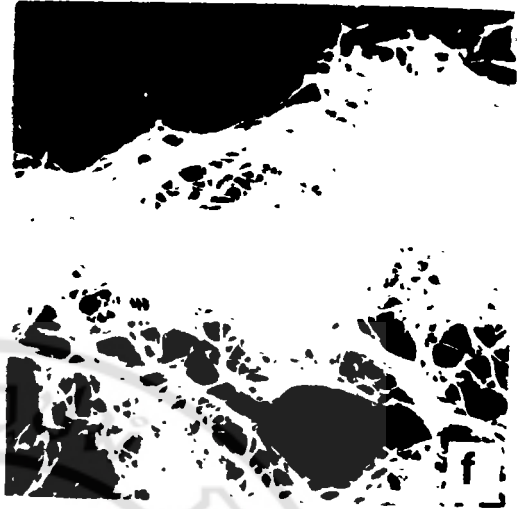


Fig. 4 Dark-field photomicrographs showing rat sperm agglutination mediated by seed extracts. Caput (b, d, f, h, j) and cauda (a, c, e, g, i) epididymal sperm were separately incubated with 0.4 mg/ml seed extracts. The extracts were : a and b, PBS (control); c and d, Jack fruit extract; e and f, red kidney bean extract; g and h, garden pea extract; i and j rambutan extract.



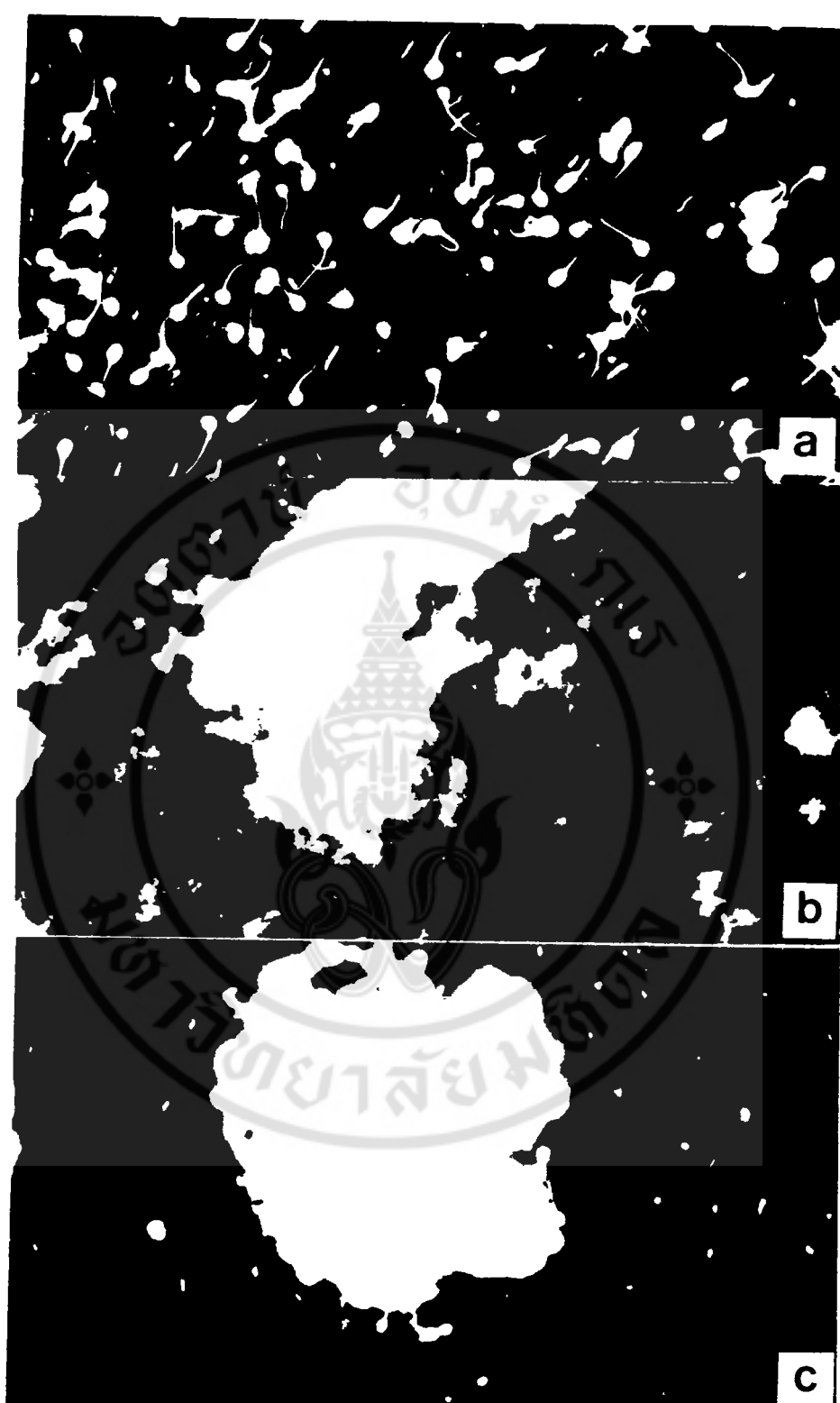


Fig 5 Dark-field photomicrographs showing human sperm agglutination mediated by 0.25 mg/ml Jack fruit extract. a = control ; b = unwashed sperm ; c = washed sperm.

Table 6 Sperm agglutination test II. Rat and human sperm were separately incubated with 0.4 mg/ml and 0.25 mg/ml seed extracts, respectively. Values shown are mean $\pm$ SE (n = 3). See

Table 1 for other details.

	<u>RCd</u>	<u>RCp</u>	<u>UHS</u>	<u>WHS</u>
1. Jack fruit	4	4	4	4
2. Red kidney bean	4	4	4	4
3. Broad bean	4	4	3.3 $\pm$ 0.6	4
4. Garden pea	4	4	3.0 $\pm$ 1.0	4
5. Sa-taw	3.0 $\pm$ 1.0	4	1.0 $\pm$ 0.6	2.6 $\pm$ 1.3
6. Yard-long bean	1.0 $\pm$ 0.5	2.0 $\pm$ 1.1	0.6 $\pm$ 0.3	1.6 $\pm$ 0.8
7. Green gram	1.3 $\pm$ 0.6	2.0 $\pm$ 1.0	1.6 $\pm$ 0.8	3.0 $\pm$ 1.0
8. Soybean	0	1.3 $\pm$ 0.6	0	0.6 $\pm$ 0.3
9. Peanut	0	2.6 $\pm$ 1.3	0.3 $\pm$ 0.3	2.3 $\pm$ 1.2

Table 7 Minimal concentration of seed extracts mediating sperm agglutination at the score of 1. Values are averages of 3 experiments. See Table 1 for other details.

<u>Seed extract</u>	<u>Minimal concentration ($\mu\text{g/ml}$)</u>			
	<u>RCd</u>	<u>RCp</u>	<u>UHS</u>	<u>WHS</u>
1. Jack fruit	0.2	0.4	25	7.8
2. Red kidney bean	6.5	3.2	30	3.9
3. Sa-taw	6.5	1.6	250	125
4. Garden pea	6.5	6.5	250	60
5. Broad bean	13	6.5	250	125
6. Yard-long bean	210	210	NE ¹	60
7. Peanut	NE ²	13	NE ¹	150
8. Soybean	NE ²	420	NE ¹	250
9. Green gram	100	100	250	250

NE¹ = No effect at 0.25 mg/ml

NE² = No effect at 0.4 mg/ml

Seed extracts were also tested for their ability to agglutinate erythrocytes. Seed extracts from balsam pear, rambutan, red kidney bean, broad bean, Jack fruit, garden pea and horse-tamarind (matured seeds) were able to mediate agglutination of human erythrocytes, untreated or neuraminidase - treated cells (Table 8). Peanut and soybean extracts mediated agglutination of neuraminidase-treated red cells only. Seed extracts from sorghum, sapodilla, job's tear and rice caused hemolysis of both untreated and neuraminidase-treated red cells. Extract from cassava roots also showed the hemolytic property. Several other seed extracts showed no hemagglutinating activity.

The comparison between sperm agglutination and hemagglutination tests is shown in Table 9. Some seed extracts, Jack fruit, red kidney bean, broad bean, and garden pea, were active in agglutinating both sperm and erythrocytes. Seed extracts from Sa-taw soybean, and yard-long bean agglutinated only rat and human sperm but not erythrocytes. Rambutan seed extract agglutinated rat caput epididymal sperm and human erythrocytes while longan seed extract reacted only with caput epididymal sperm. Seed extract from tamarind could agglutinate rat caput and cauda epididymal sperm and human erythrocytes but not human sperm. Balsam pear seed extract did not agglutinate sperm but agglutinated human red blood cells.

Table 8 Hemagglutination test. Human erythrocytes, untreated and neuraminidase-treated were incubated with seed extracts at room temperature for 1 h. Hemagglutination was observed macroscopically and scored as described in section 2.9.3. Mean $\pm$ SE for n = 6 was calculated. Seed extracts under "No hemagglutination" did not mediate hemagglutination. Seed extracts under "Hemolysis" caused hemolysis.

Table 8

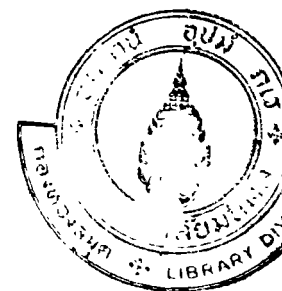
Seed extracts	Protein (mg/ml)	Hemagglutination	
		Untreated HRBC	Neuraminidase -treated HRBC
Balsam pear	0.1	4.0±0.0	4.0±0.0
Rambutan	1.0	4.0±0.0	4.0±0.0
Red kidney bean	2.0	4.0±0.0	4.0±0.0
Broad bean	1.1	3.2±0.2	4.0±0.0
Jack fruit	2.5	3.0±0.0	3.0±0.0
Garden pea	0.5	2.6±0.4	2.1±0.4
Horse-tamarind, matured	0.6	1.0±0.0	1.0±0.0
Peanut	0.3	0	4.0±0.0
Soybean	1.8	0	3.0±0.0

Table 8 (continued)

<u>No hemagglutination</u>		<u>Hemolysis</u>
Green gram	Salacca	Sorghum, dotted
Yard-long bean	Litchi	Sapodilla
	Lotus	Cassava, root
Horse-tamarind, immatured	Corn	Sorghum, red
Papaya	Sunflower	Job's tear
Jamaica Sorrel	Sa-taw	Rice
Custard apple	Black gram	
Winged bean	Longan	
Lentil	Tamarind	

Table 9 Comparison between sperm agglutination and hemagglutination tests (Data obtained from Table 5, 6, and 8)

<u>Seed extract</u>	<u>Agglutination</u>			<u>Untreated-HRBC</u>
	<u>RCd</u>	<u>RCp</u>	<u>WHS</u>	
1. Jack fruit	+	+	+	+
2. Red kidney bean	+	+	+	+
3. Broad bean	+	+	+	+
4. Garden pea	+	+	+	+
5. Sa-taw	+	+	+	-
6. Yard-long bean	+	+	+	-
7. Soybean	+	+	+	-
8. Tamarind	+	+	-	+
9. Rambutan	-	+	-	+
10. Longan	-	+	-	-
11. Balsam pear	-	-	-	+



3.2 Effect of seed extracts on initiation of sperm motility

Spermatozoa in the cauda epididymidis of rat are non-motile. Initiation of caudal sperm motility can be induced by adding of a salt solution such as 0.9% NaCl (Chulavatnatol, 1982). In this study, phosphate buffer saline extracts of seeds were tested for their ability to inhibit initiation of sperm motility in cauda epididymal sperm (see details of experiment in section 2.12). Jack fruit extract was found to completely inhibit the initiation of sperm motility (Table 10). Extracts from red kidney bean and garden pea also showed strong inhibitory effect while several seed extracts partially inhibited the motility initiation. Seed extracts from corn, rice, Sa-taw, and peanut did not have any significant effect. Since the inhibition test was not done using the same protein concentration of the extracts, strict comparison of the potencies among the extracts cannot be made. Comparison of inhibiting potencies can be made among certain extracts with comparable protein concentrations.

Table 10 Effect of seed extracts on inhibition of sperm mobility.

The motility initiation was performed as described in section 2.12. Mean $\pm$ SE for n = 6 was calculated.

<u>Protein extract</u>	<u>Protein (mg/ml)</u>	<u>Sperm motility initiation (X $\pm$ SE)</u>
Control (PBS)		4.0 $\pm$ 0.0
1. Jack fruit	2.5	0.0 $\pm$ 0.0
2. Red kidney bean	2.0	0.6 $\pm$ 0.2
3. Garden pea	0.5	0.8 $\pm$ 0.3
4. Rambutan	1.0	1.0 $\pm$ 0.3
5. Green gram	3.7	1.2 $\pm$ 0.7
6. Broad bean	1.1	1.4 $\pm$ 0.4
7. Balsam pear	0.1	1.6 $\pm$ 0.6
8. Sorghum, dotted	0.07	2.0 $\pm$ 0.4
9. Potato	1.0	2.0 $\pm$ 0.8
10. Tamarind	0.18	2.4 $\pm$ 0.2
11. Lotus	0.003	2.4 $\pm$ 0.2
12. Jamaica sorrel	0.08	2.4 $\pm$ 0.4
13. Horse-tamarind, im- matured	0.001	2.4 $\pm$ 0.5
14. Job's tear	0.002	2.4 $\pm$ 0.6
15. Yard-long bean	0.16	2.4 $\pm$ 0.6

Table 10 (continued)

<u>Protein extract</u>	<u>Protein (mg/ml)</u>	<u>Sperm motility initiation (X ± SE)</u>
Control (PBS)		4.0±0.0
16. Cassava	0.01	2.6±0.6
17. Salacca	0.02	2.6±0.6
18. Sapodilla	0.15	2.6±0.6
19. Soybean	1.8	2.8±0.4
20. Litchi	0.01	3.0±0.4
21. Custard apple	0.04	3.0±0.4
22. Lentil	3.13	3.2±0.3
23. Longan	0.24	3.2±0.3
24. Durian	0.12	3.2±0.2
25. Sorghum, red	0.03	3.4±0.2
26. Black gram	0.9	3.4±0.2
27. Horse-tamarind, matured	0.6	3.4±0.4
28. Sunflower	0.6	3.4±0.4
29. Winged bean	0.01	3.6±0.2
30. Peanut	0.3	3.6±0.4
31. Sa-taw	0.27	3.8±0.2
32. Rice	0.05	4.0±0.0
33. Corn	0.13	4.0±0.0

3.3 General properties of Jack fruit lectin

From the survey study, Jack fruit seeds were considered to have interesting and potent agglutinating agent or lectin. Further investigations on the Jack fruit seed extract were carried out.

3.3.1 Tissue distribution

Jack fruit seeds were excised with a knife into three macroscopically distinct parts: the outer, middle, and inner parts as shown in Fig. 6. Each part was homogenized and protein was extracted by ammonium sulfate precipitation as described in section 2.7. Sperm agglutination and hemagglutination was tested and the specific activity (units/mg protein) was determined as described in section 2.9.4. The result shown in Table 11 indicated that all three parts of Jack fruit seed contained the agglutinating agents. The specific activity was high in the outer and inner parts. Since the inner part contained most protein and agglutinating activity, it was considered not worthwhile to peel off the outer and the middle layer of the seeds but rather the whole seeds were extracted in subsequent studies.

3.3.2 Ammonium sulfate fractionation

Whole Jack fruit seeds were extracted and fractionated by increasing saturation of ammonium sulfate. Fig. 7 shows specific activity (units/mg protein) of protein precipitated by various concentration of ammonium sulfate. The amount of protein (g/kg seeds) in each fractions is shown in Fig. 7 b. A fraction precipitated by 50-60% saturation of ammonium sulfate (later called "S-60") was found

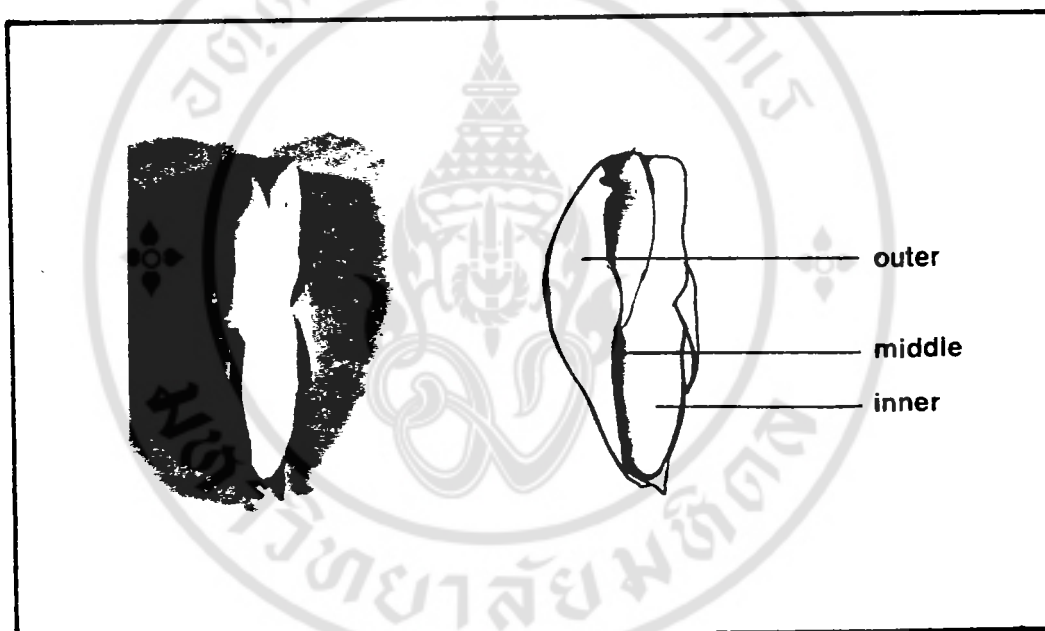


Fig. 6 Cross section of Jack fruit seeds. There are three macroscopically distinct parts (shown in the diagram) : the outer, middle, and inner parts.

Table 11 Distribution of Jack fruit lectin. A seed of Jack fruit is composed of three parts: outer, middle, and inner part. The three parts were homogenized separately and then proteins were precipitated by 60% saturation of $(\text{NH}_4)_2\text{SO}_4$. Sperm agglutination mediated by each extracts was tested as previously.

Part of seed	Protein (mg)	Specific activity ($\times 10^{-4}$)			
		RCd	RCp	WHS	RBC
outer	0.5	0.8 $\pm$ 0.2	3.4 $\pm$ 0.8	1.3 $\pm$ 0.0	41.0 $\pm$ 0.0
middle	0.3	0.4 $\pm$ 0.1	1.4 $\pm$ 0.3	0.5 $\pm$ 0.0	0.0 $\pm$ 0.0
inner	19.0	0.7 $\pm$ 0.2	2.2 $\pm$ 0.0	1.1 $\pm$ 0.0	34.0 $\pm$ 0.0

(Values are mean $\pm$ SE, for n = 3)

to contain the highest total activity (specific activity x protein concentration). Based on specific activities, there seemed to be segregation of hemagglutinating activity in the range of 50-80% ammonium sulfate saturation while sperm agglutinating activities were found mainly at a lower saturation range (50-70%).

3.3.3 Thermal stability

Since the S-60 fraction contained most of the agglutinating activities and would be used in further study, it was necessary to know the stability of the active agents in this fraction. So S-60 fraction was incubated at 37°C for 0, 30, 60, 120, and 180 min. No loss of protein or agglutinating activities (units/ml) was observed within a period of 2 h (Fig. 8).

3.3.4 Sugar specificity

Lectins normally agglutinate cells by binding with specific sugar on the cell surface. One indirect but convenient way to show that the agglutinating agent in the Jack fruit extract binds a specific sugar is to find a sugar that can inhibit the agglutination.

Various sugars were tested for their ability to inhibit agglutination according to the methods in section 2.10. N-Acetyl-galactosamine (75 mM) could inhibit 100% rat sperm agglutination and it can completely inhibit agglutination of unwashed and washed human sperm at 50 and 25 mM, respectively (Table 12). For red blood cells, 30 mM of GalNAc was found to inhibit 50% hemagglutination. Galactose, at the concentration higher than GalNAc, also inhibited human sperm agglutination and hemagglutination. Other sugar listed in Table 12,

at the concentration of 0.2 M, could not inhibit sperm agglutination and hemagglutination. Hemolysis was observed when n-octy- β -D-glucopyranoside was tested. The finding above indicated that Jack fruit lectin was specific for GalNAc.



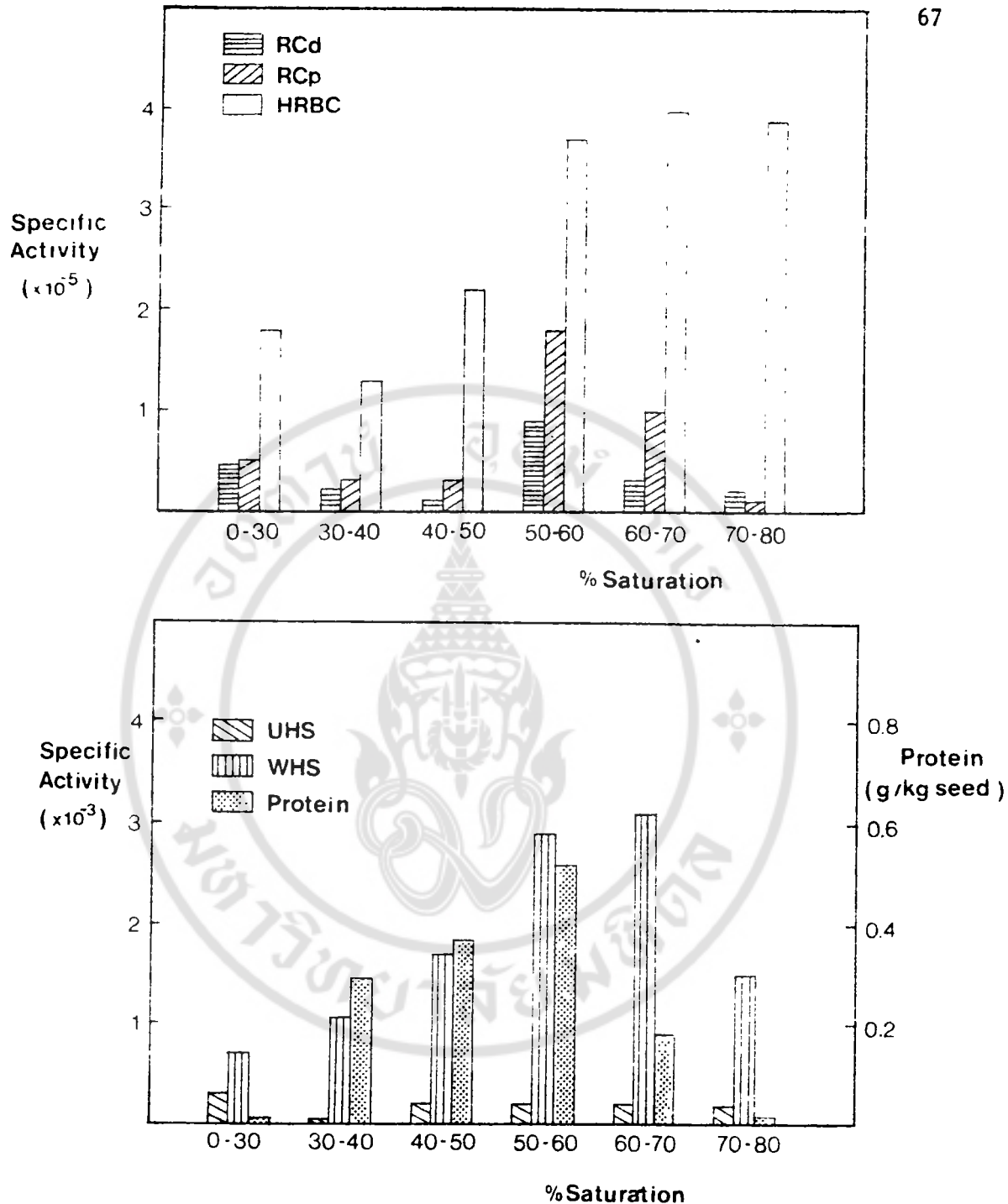


Fig. 7 Distribution of Jack fruit lectin precipitated at different saturation of ammonium sulfate. Jack fruit seeds were homogenized and centrifuged to remove insoluble materials as described in section 2.7. Protein fractions were obtained from the extract by sequential precipitation by adding the solid salt with increasing % saturation. (The values are averages of three experiments).

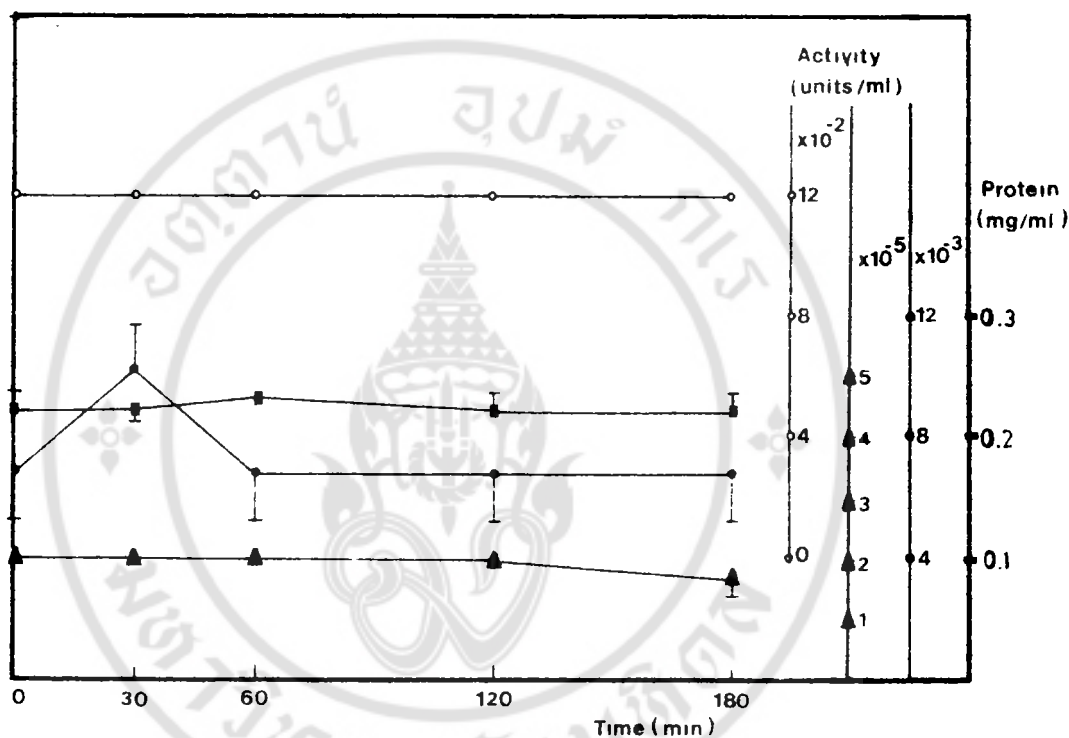


Fig 8 Stability of Jack fruit extract. Jack fruit extracts (S-60 fraction) was divided into 5 aliquots and incubated at 37°C for 0, 30, 60, 120, and 180 min. At each time point, a sample of 50 μ l was withdrawn, sperm agglutination and hemagglutination were tested and activity (units/ml) was determined as previously. The result shows caput epididymal sperm (—●—), washed human sperm (—○—) agglutination, hemagglutination (—▲—) and protein concentration (—■—). ($\bar{X} \pm SE$, $n = 3$)

Table 12 Sugar specificity of Jack fruit lectin. Jack fruit extract (S-60) was diluted to the lowest concentration that mediated sperm agglutination at the score 4 (or 2 for hemagglutination). Upto 200 mM of each sugar was added in the agglutination inhibition tests as described in section 2.10. The amount of sugar shown are those required for 100% inhibition of sperm agglutination and 50% inhibition of hemagglutination.

Table 12

<u>Sugar</u>	<u>mM sugar</u>				
	<u>RCD</u>	<u>RCp</u>	<u>UHS</u>	<u>WHS</u>	<u>HRBC</u>
GalNAc	75	75	50	25	30
Gal	NI	NI	100	100	100
n-octyl-β-D-glucopyranoside	200	200	NI	NI	H

NI : No inhibition at 200 mM

H : Hemolysis at 200 mM

Sugars(0.2 M) showing no inhibition of agglutination by seed extract of Jack fruit.

Glc	Man	Fructose
GlcNH ₂	ManNH ₂	Fucose
GlcNAc	ManNAc	Sucrose
2-Deoxy-D-Glc		Xylose
3-O-CH ₃ -Glc		Rhamnose

3.4 Purification of Jack fruit lectin

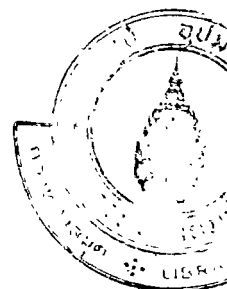
Based on the finding that Jack fruit lectin bound specifically to GalNAc (section 3.3.4), affinity chromatography on resin-bound GalNAc was tried in order to purify the lectin.

3.4.1 Purification by GalNAc-Sepharose column

GalNAc was coupled to Sepharose as described in section 2.13 and a S-60 fraction was applied and affinity chromatography was performed as in section 2.14.1. As shown in Fig. 9, Jack fruit lectin bound to GalNAc-Sepharose was eluted with 0.1 M GalNAc in PBS. From one of several runs, the protein concentration, specific activity, total activity, protein recovery and activity recovery are shown in Table 13. It was observed that the unbound fraction still contained agglutinating activity. When the unbound fraction was re-applied onto the regenerated column, more activity was recovered in the bound fraction, suggesting an overloading of the first chromatography. As the recoveries of protein and activity were rather low, another type of resin-bound sugar was tried.

3.4.2 Purification by Affigel-galactosamine column

Affigel-galactosamine, the commercially available adsorbent consisting of cross-linked agarose coupled with N-acetyl-galactosamine, was used to purify Jack fruit lectin according to procedure in section 2.14.2. Jack fruit lectin bound to column was eluted with 0.03 M galactose in PBS (Fig. 10). Galactose at this concentration was found to elute all of bound proteins, since no protein was further eluted out when the column was sequentially washed with 0.1 M borate



buffer pH 8.5 containing 0.5 M NaCl followed by 0.1 M acetate buffer pH 4.5 containing 0.5 M NaCl.

The amount of Jack fruit lectin, the activity and specific activity were shown in Table 14. There was no significant loss of activity into the unbound fraction and good recoveries of protein and activity in the bound fraction were evident. Since less than 10% of protein was removed into the unbound fraction, the specific activity in the bound fraction was not significantly improved over that in S-60.

The bound fraction from the Affigel-galactosamine column was used in further studies and shown to be "purified" Jack fruit lectin.

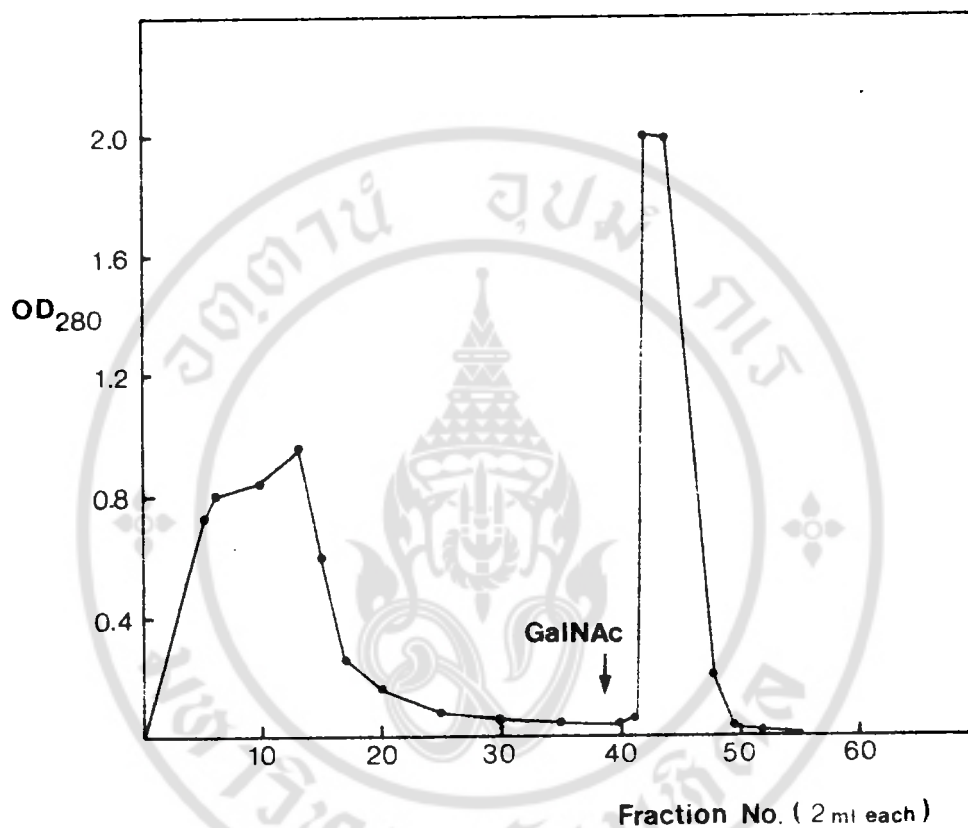


Fig. 9 Protein profile of Jack fruit extract eluted from a column of GalNAc-Sepharose. Epoxy-activated Sepharose 6B was coupled with GalNAc as in section 2.13 and packed into a column (1.5 x 1.6 cm). After equilibration with PBS, Jack fruit extract (S-60), 7.5 mg in 20 ml PBS, was pumped into column at flow rate 10 ml/h. The eluate was monitored at 280 nm. The unbound proteins were washed from the column with PBS and then bound protein was eluted with 0.1 M GalNAc in PBS (see details in section 2.14.1).

Table 13 Purification of Jack fruit lectin. Jack fruit lectin was purified by affinity chromatography on a column of GalNAc-Sepharose (Fig. 9). The unbound and bound fractions were pooled and dialyzed against PBS. The protein concentration was assayed as in section 2.8 and the activity was determined as in section 2.9.4.

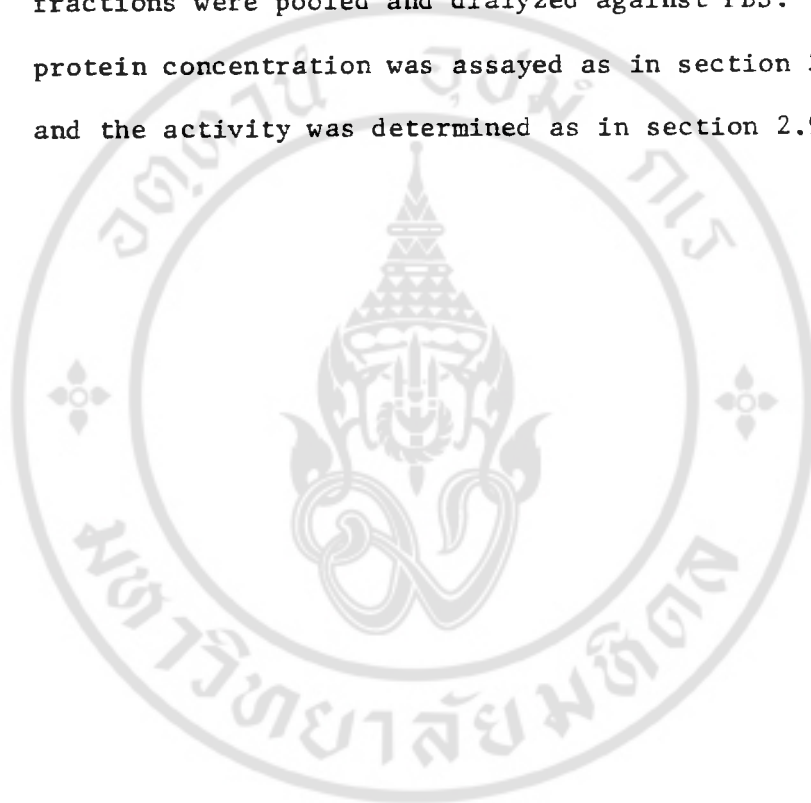


Table 13

<u>Fraction</u>	<u>Protein</u> <u>conc.</u>	<u>Total</u> <u>protein</u>	<u>Protein</u> <u>recovery</u>	<u>Specific</u> <u>activity</u>	<u>Total</u> <u>activity</u>	<u>Activity</u> <u>recovery</u>
	(mg/ml)	(mg)	(%)	($\times 10^{-5}$) (units/mg)	($\times 10^{-6}$) (units)	(%)
<u>RCp</u>						
S-60	1.9	7.5	100.0	0.7	0.5	100.0
unbound	0.04	2.0	26.7	0.3	0.1	12
sugar-eluted	0.18	3.6	48.0	1.1	0.4	78
<u>WHS</u>						
S-60	1.9	7.5	100.0	0.2	0.2	100.0
unbound	0.04	2.0	26.7	0.2	0.03	18.8
sugar-eluted	0.18	3.6	48.0	0.3	0.1	62.5
<u>HRBC</u>						
S-60	1.9	7.5	100.0	3.4	2.6	100.0
unbound	0.04	2.0	26.7	4.2	0.8	30.8
sugar-eluted	0.18	3.6	48.0	4.5	1.6	61.5

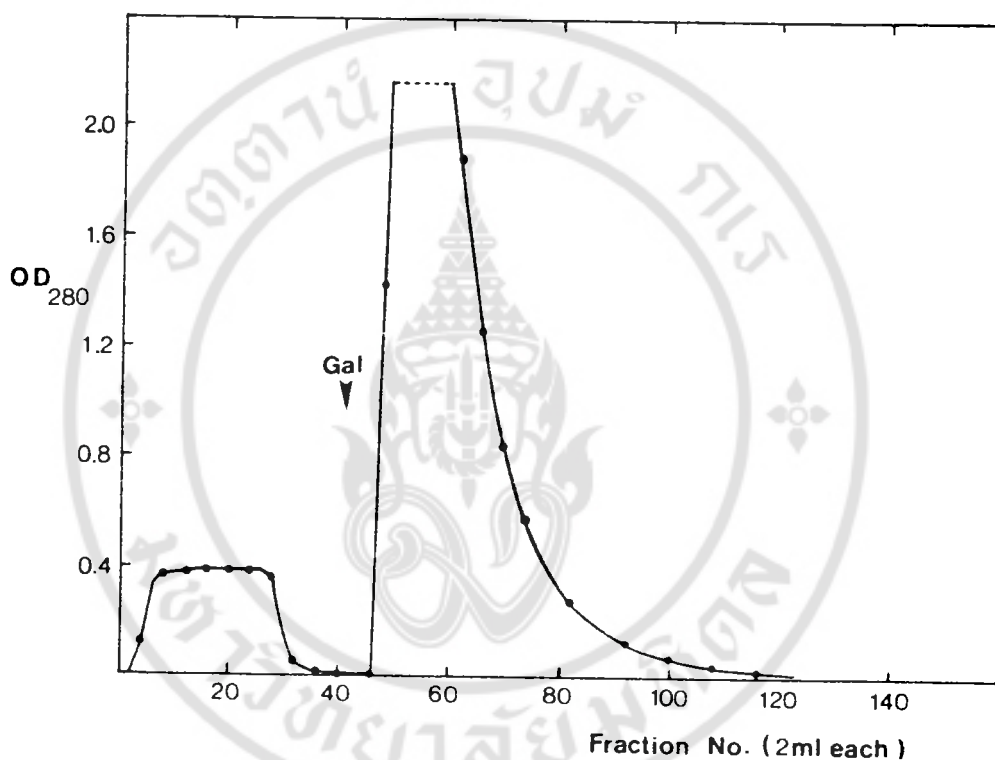


Fig. 10 Protein profile of S-60 eluted from a column of Affigel-galactosamine. S-60 was passed through Affigel-galactosamine column (1.3 x 4.3 cm). The unbound proteins were washed by PBS and then bound proteins were eluted with 0.03 M Gal in PBS. The unbound and bound fractions were pooled and dialyzed against PBS. The protein profile was made by measuring the absorbance of the eluate at 280 nm.

ADDENDUM

From the result shown in Fig. 7, total protein and total activity of crude extract were determined by summation of those of each fractions. Protein concentration and specific activity were then calculated and shown below.

<u>Protein</u> <u>conc.</u> (mg/ml)	<u>Total</u> <u>protein</u> (mg)	<u>Specific</u> <u>activity</u> x 10 ⁻⁵ (units/mg)	<u>Total</u> <u>activity</u> x 10 ⁻⁶ (units)
2.2	1,400	<u>HRBC</u> 2.8	390
		<u>WHS</u> 0.02	3
		<u>RCp</u> 0.9	130

Table 14 Purification of Jack fruit lectin by Affigel-galactosamine.

	<u>Protein</u> <u>conc.</u>	<u>Total</u> <u>protein</u>	<u>Protein</u> <u>recovery</u>	<u>Specific</u> <u>activity</u>	<u>Total</u> <u>activity</u>	<u>Activity</u> <u>recovery</u>
	(mg/ml)	(mg)	(%)	($\times 10^{-5}$) (units/mg)	($\times 10^{-6}$) (units)	(%)
<u>HRBC</u>						
S-60	2.6	20.5	100.0	3.4	7.0	100.0
unbound	0.7	1.7	8.3	0.01	0.02	0.3
sugar-eluted	0.1	18.2	88.8	3.7	6.7	95.7
<u>WHS</u>						
S-60	2.6	20.5	100.0	0.1	0.2	100.0
unbound	0.7	1.7	8.3	0.003	0.01	2.2
sugar-eluted	0.1	18.2	88.8	0.1	0.3	108.6
<u>RCp</u>						
S-60	2.6	20.5	100.0	1.1	2.2	100.0
unbound	0.7	1.7	8.3	0.1	0.2	9.3
sugar-eluted	0.1	18.2	88.8	0.9	1.7	79.1

3.5 Characterization of Jack fruit lectin

3.5.1 Molecular weight determination by Sephadex G-200

Jack fruit lectin purified from Affigel-galactosamine column was subjected to gel filtration in a column of Sephadex G-200 equilibrated with PBS. The protein was eluted from the column with PBS and the protein profile was shown in Fig. 11. Only one peak of protein came out of the column and the molecular weight was determined to be 42,000 based on a standard curve constructed from the elution of molecular weight markers (Fig. 11, Fig. 12, and Fig. 13). The activity in fractions containing protein was determined. It was noted that the protein peak was approximately symmetrical. The profile of hemagglutinating and sperm agglutinating activities were each symmetrical and coincided with the protein profile. Thus, the bound fraction from the Affigel-galactosamine column was considered as "purified Jack fruit lectin".

3.5.2 Determination of subunit molecular weight by SDS-PAGE

Purified Jack fruit lectin was subjected to SDS-PAGE (Fig. 14). The lectin was found to compose of two subunits of which molecular weights were 13,000 and 18,000 (Fig. 15). The smaller subunit was more intensively stained than the larger one. Since the molecular weight of non-denatured Jack fruit lectin obtained from Sephadex G-200 is 42,000, the structure of Jack fruit lectin may compose of three subunits, two subunits of MW 13,000 and one subunit of MW 18,000. SDS-PAGE of S-60 (Fig. 14) showed 3 protein bands of MW 29,500, 13,000, and 18,000.

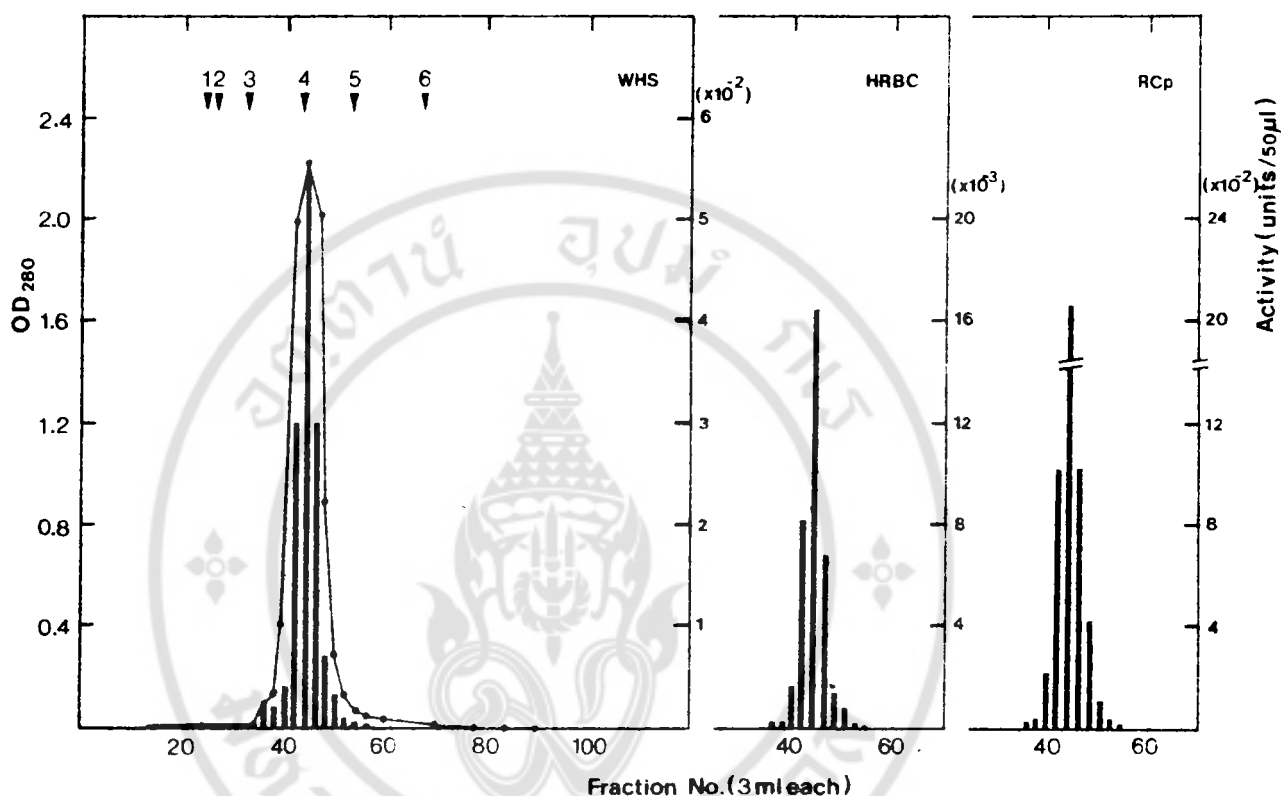


Fig. 11 Protein profile of Jack fruit lectin eluted from a column of Sephadex G-200. Jack fruit lectin (10 mg in 2 ml PBS) purified from Affigel-galactosamine column was passed through Sephadex G-200 column (1.6 x 90 cm) previously equilibrated with PBS. The protein (OD at 280 nm) was eluted from the column with PBS. The agglutination activity (units/50 μ l) of indicated fractions was plotted as histograms. The arrowheads indicate the position of markers : 1, blue dextran (MW 2,000,000); 2, ferritin (MW 440,000); 3, aldolase (MW 158,000); 4, ovalbumin (MW 43,000); 5, ribonuclease A (MW 13,700); 6, DNP-lysine (MW 312).

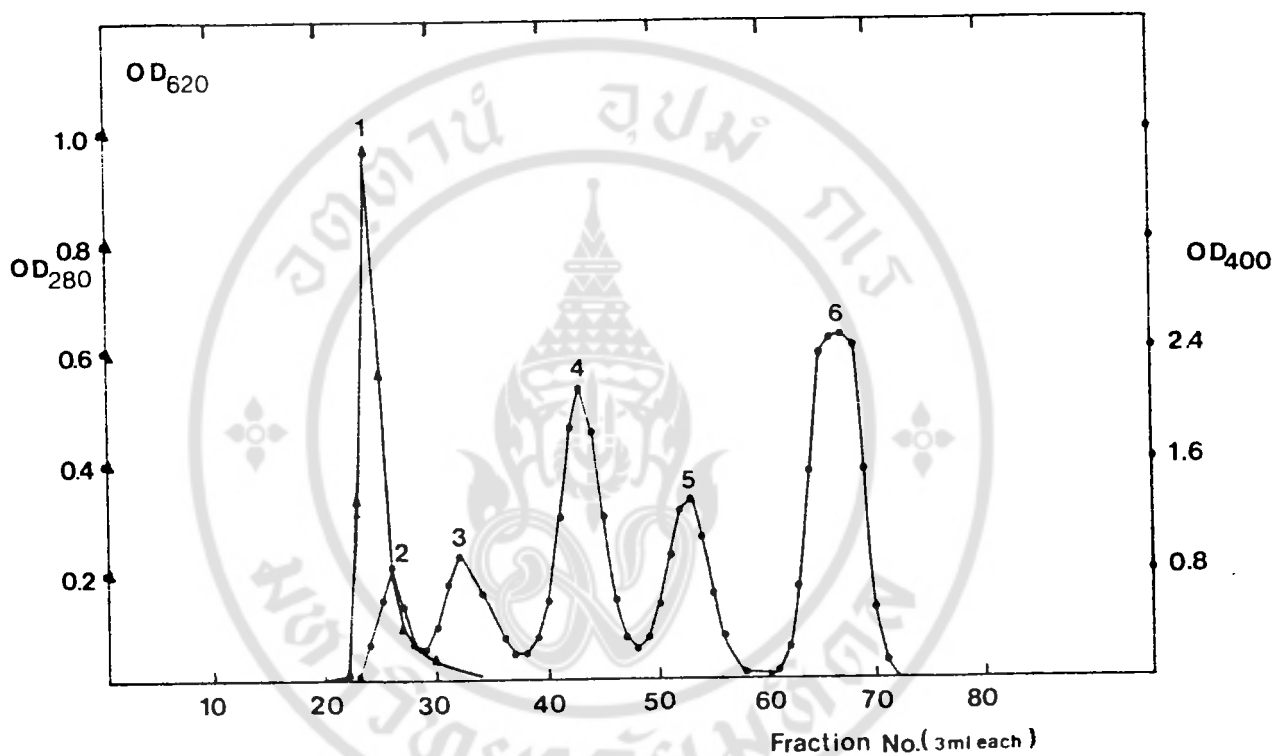


Fig. 12 Standard proteins and dye markers eluted from a Sephadex G-200 column. The absorbance of blue dextran and DNP-lysine were measured at 620 and 400 nm, respectively. For other standard proteins the absorbance was measured at 280 nm. 1 = blue dextran, 2 = ferritin, 3 = aldolase, 4 = ovalbumin, 5 = ribonuclease A, 6 = DNP-lysine.

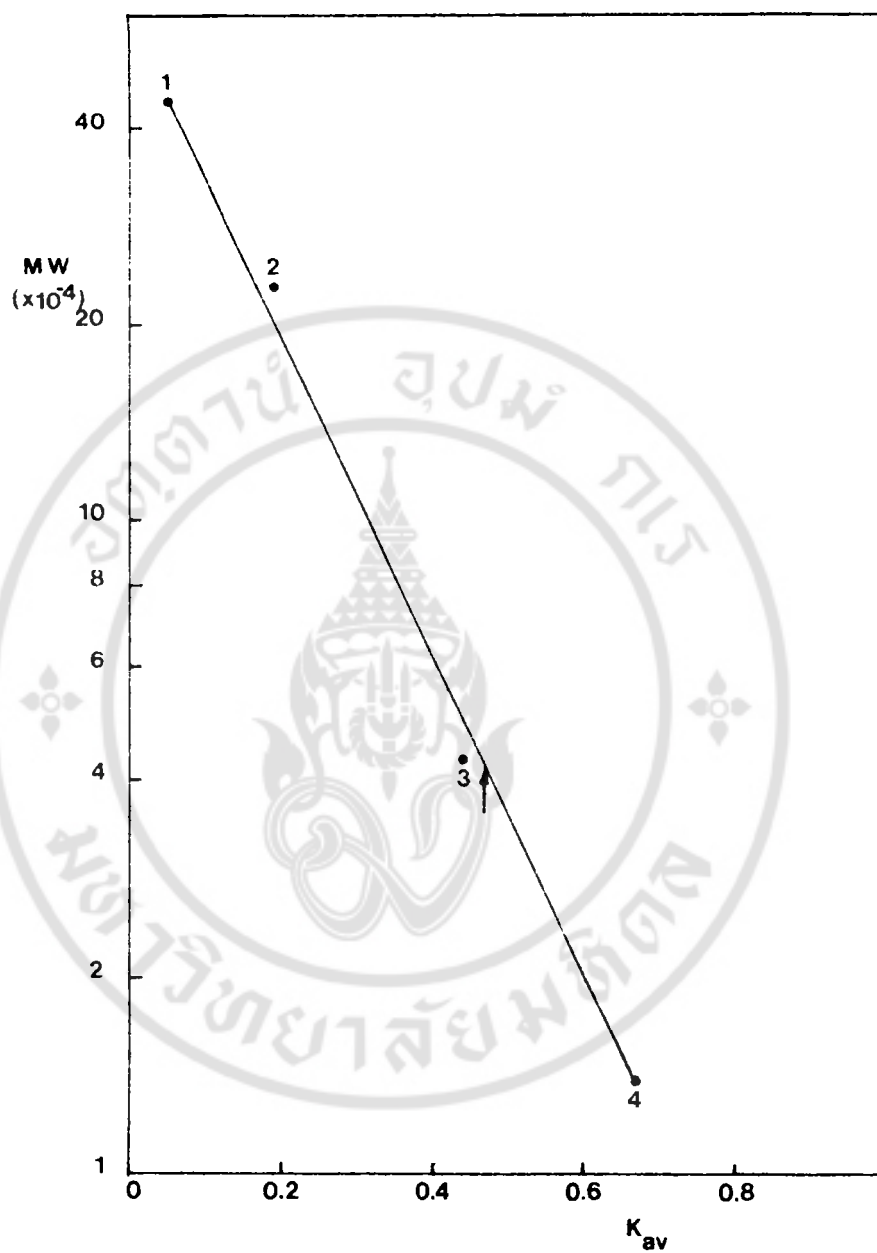


Fig. 13

A standard curve for the determination of molecular weight of Jack fruit lectin by Sephadex G-200. The data are shown in Fig. 11 and Fig. 12. Log MW of standard proteins were plotted against their corresponding K_{av} . The K_{av} of Jack fruit lectin was indicated as an arrow. The standard proteins are : 1, ferritin; 2, aldolase; 3, ovalbumin; 4, ribonuclease A.

3.5.3 Heterogeneity of Jack fruit lectin on ND-PAGE

S-60, purified Jack fruit lectin, and unbound protein from Affigel-galactosamine were subjected to non-denaturing polyacrylamide gel electrophoresis. There was no common bands between the unbound and the bound fractions (Fig. 16, a). Jack fruit lectin (the bound fraction) which was found to be homogeneous in Sephadex G-200 (Fig. 11) showed charge heterogeneity on ND-PAGE. The lectin protein showed a smear pattern and the activity appeared to spread over the protein smear and formed two broad activity peaks (Fig. 16, b).

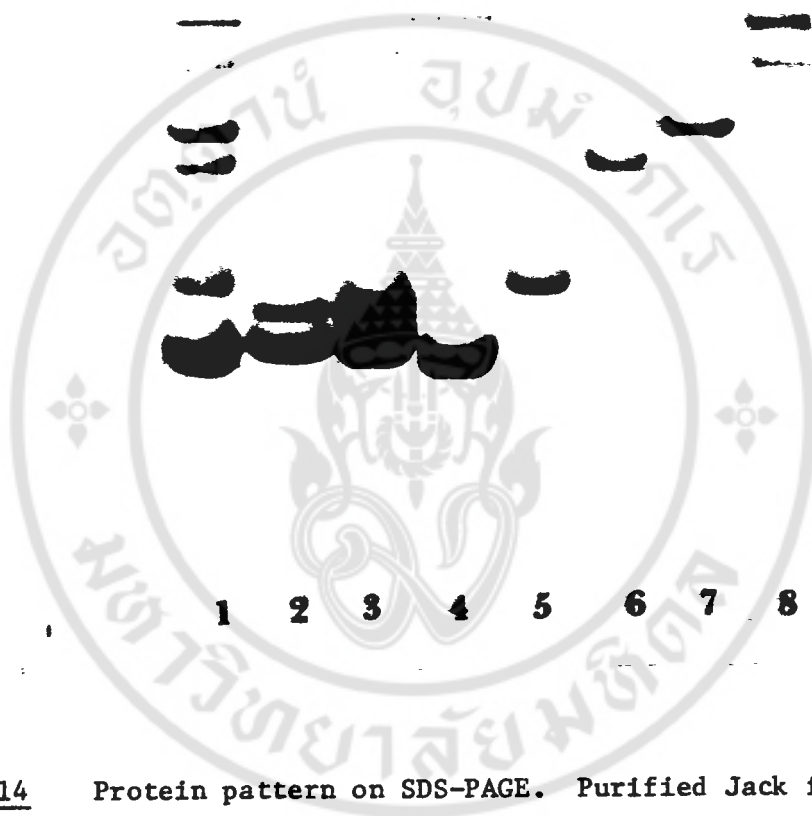


Fig. 14 Protein pattern on SDS-PAGE. Purified Jack fruit lectin and S-60 were subjected to SDS-PAGE according to Anderson et al (1983). Lane 1, mixed standard proteins : phosphorylase b (MW 94,000), BSA (MW 68,000), ovalbumin (MW 43,000), soybean trypsin inhibitor (MW 20,000), and cytochrome c (MW 12,300); Lane 2, Jack fruit lectin; Lane 3, S-60 ; Lane 4, cytochrome c; Lane 5, soybean trypsin inhibitor; Lane 6, ovalbumin; Lane 7, BSA; Lane 8, phosphorylase b.

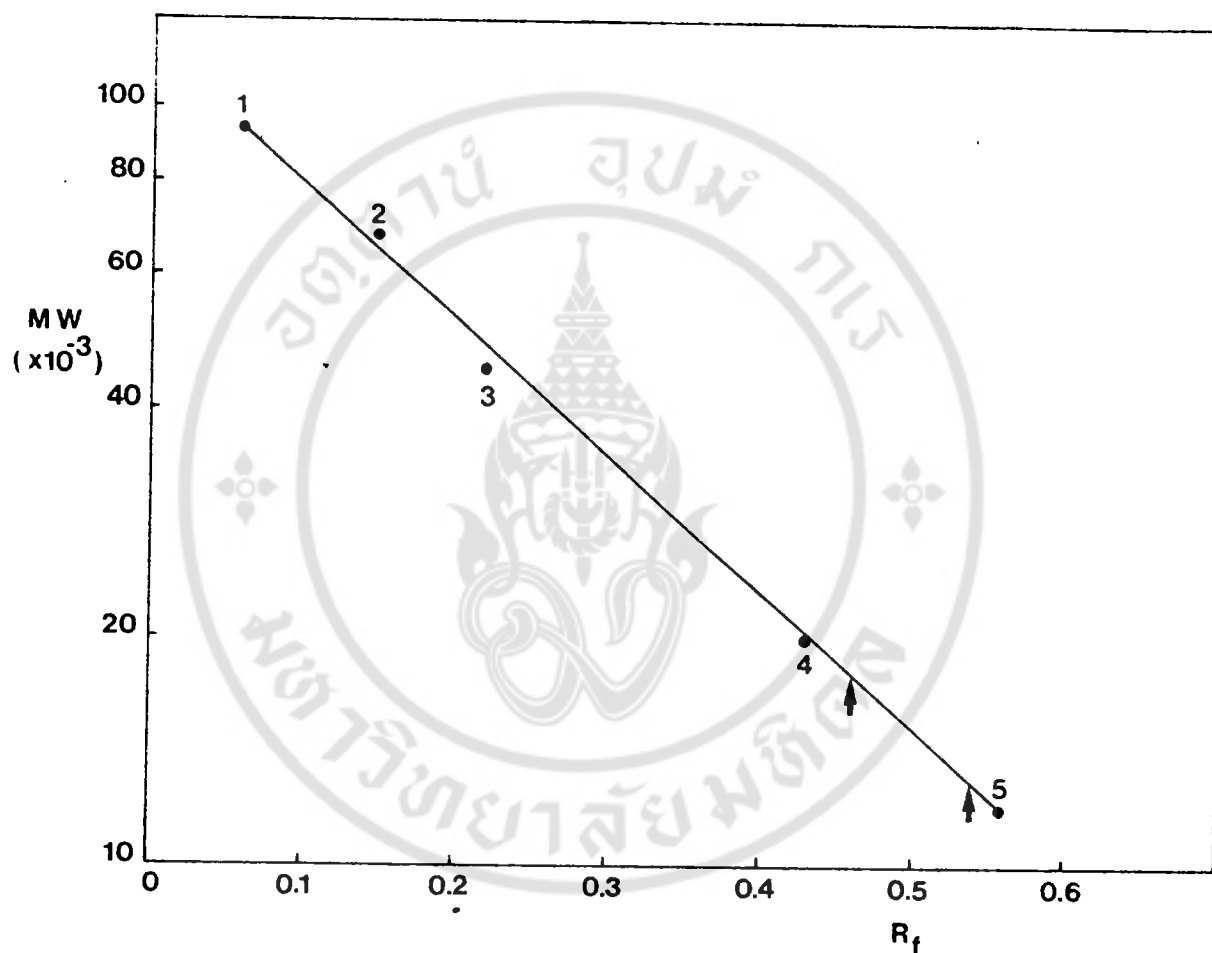


Fig. 15 Standard curve for the determination of subunit molecular weights of Jack fruit lectin by SDS-PAGE. The relative mobility of standard proteins, the average of 3 gels, was plotted against corresponding log MW. The standard proteins are : 1, phosphorylase b; 2, BSA; 3, Ovalbumin; 4, soybean trypsin inhibitor; 5, cytochrome c.

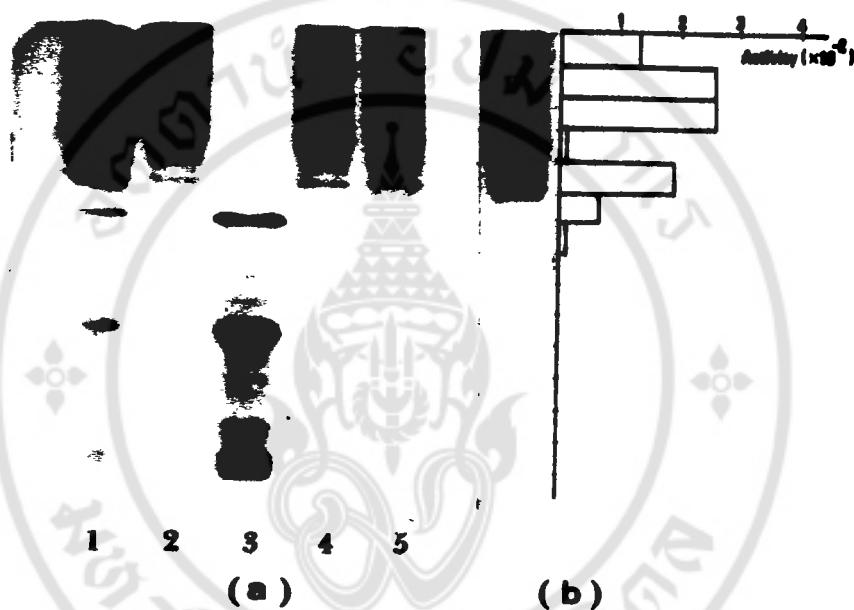


Fig. 16 Non-denaturing polyacrylamide gel electrophoresis of purified Jack fruit lectin (a) Lane 1, 100 μ g of S-60; Lane 2, 50 μ g of S-60; Lane 3, 40 μ g of unbound proteins from Affigel-galactosamine column; Lane 4, 40 μ g of purified Jack fruit lectin; Lane 5, 50 μ g of purified Jack fruit lectin. (b) 60 μ g of purified Jack fruit lectin. The polyacrylamide gel was sliced into 5 mm/slice. Each slice was placed into 0.5 ml PBS to elute the protein. Hemagglutination was tested and hemagglutination activity/50 μ l was shown.

3.5.4 Sensitivity of Jack fruit lectin to pronase digestion

Purified Jack fruit lectin and S-60 were incubated with pronase. The concentration of substrate and enzyme were 0.2 mg/ml (in PBS pH 7.4). The result in Table 15 indicated that purified Jack fruit lectin was sensitive to pronase whereas S-60 was resistant to this enzyme. After pronase treatment, the purified lectin still showed some hemagglutinating activity but none of the sperm agglutinating activities.

3.5.5 Immunodiffusion of Jack fruit lectin

Immunodiffusion was performed in 1% Noble agar using antibodies to lectins commercially available. Purified Jack fruit lectin was found to cross-react with anti-*Maclura pomifera* lectin (Fig. 17,b)

In comparison with S-60 fraction, immunodiffusion of purified Jack fruit lectin showed a sharp precipitin band (Fig. 18, a), suggesting its homogeneity. Unbound protein from Affigel-galactosamine column which contain no agglutination activity (Table 14) did not form precipitin band with the antibody (Fig. 18, a). In contrast, unbound protein from GalNac-Sepharose column which contained agglutination activity could react with antibody.

The precipitin bands in the agar were the result of the interaction between antigen (lectin) and antibody, not between the lectin and carbohydrate moieties of the antibody since addition of the lectin-specific sugar (0.03 M GalNac) could not reverse this reaction (Fig. 18, b).

3.5.6 Patterns of sperm agglutination

The sperm agglutination mediated by the purified Jack fruit lectin showed mixed patterns : head-to-head, head-to-tail, and tail-to-tail (Fig. 15), suggesting the possible spread of GalNAc all over the surface of sperm. It should, however, be noted that no attempt was made to quantitate the relative distributions of these patterns. In fact the patterns could be observed only in the case of partial agglutination (using low lectin concentration). It was also difficult to differentiate between true cell agglutination and simple cell-cell touching or overlapping.

Table 15 Sensitivity of Jack fruit lectin to pronase treatment.

Purified Jack fruit lectin (0.2 mg/ml) and also S-60 (0.2 mg/ml) were incubated with 0.2 mg/ml pronase in PBS pH 7.4 at 37°C for 16 h as described in section 2.17. The incubation mixtures were then 10-fold diluted with PBS. The aliquots from diluted mixtures were tested for the ability to agglutinate sperm and red cells. Agglutination and scoring are the same as in section 2.9.

Table 15

	HRBC [*] agglutination		RCp ^{**} agglutination		WHS ^{**} agglutination	
	control	pronase -treated	control	pronase -treated	control	pronase -treated
S-60	4	4	4	4	4	4
Purified Jack fruit lectin	4	1.5±0.5	4	0	4	0

Values are $\bar{X} \pm SE$

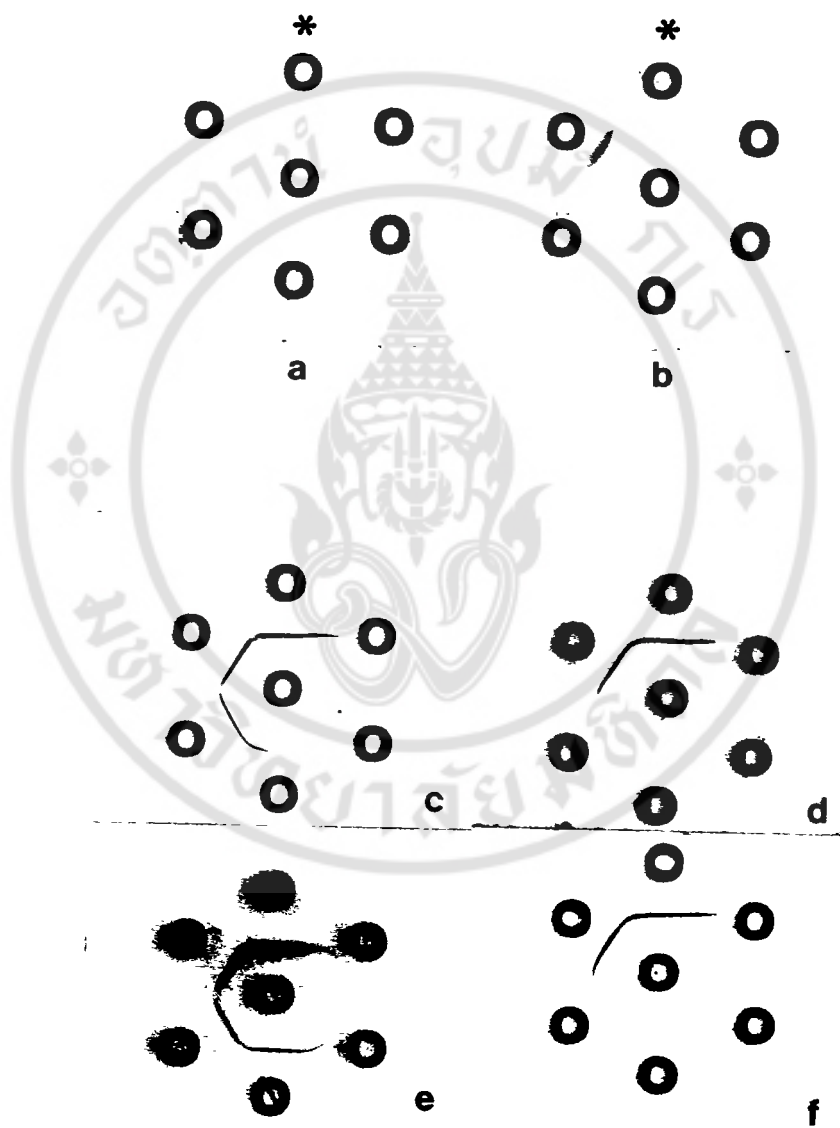
* N = 4

** N = 3

Fig. 17 Immunodiffusion of Jack fruit lectin, (a) and (b) purified Jack fruit lectin (1 μ g) was loaded into center wells and antibodies to lectins were loaded into surrounding wells. The antibodies were (starting from asterisk and turn counterclockwise) : (a) anti-PNA, anti-ConA, anti-WGA, anti-PHA, anti-TPL, anti-SBA; (b) anti-DBA, anti-MPL.

(c) anti-WGA; (d) anti-ConA; (e) anti-PNA, and (f) anti-PHA, each was 2-fold serially diluted and loaded into outer wells; the corresponding antigens (1 μ g) were loaded into center wells

anti-TPL = anti-*Tetragonolabus purpureum* lectin
 anti-SBA = anti-soybean lectin
 anti-DBA = anti-*Dolichos biflorus* lectin
 anti-MPL = anti-*Maclura pomifera* lectin



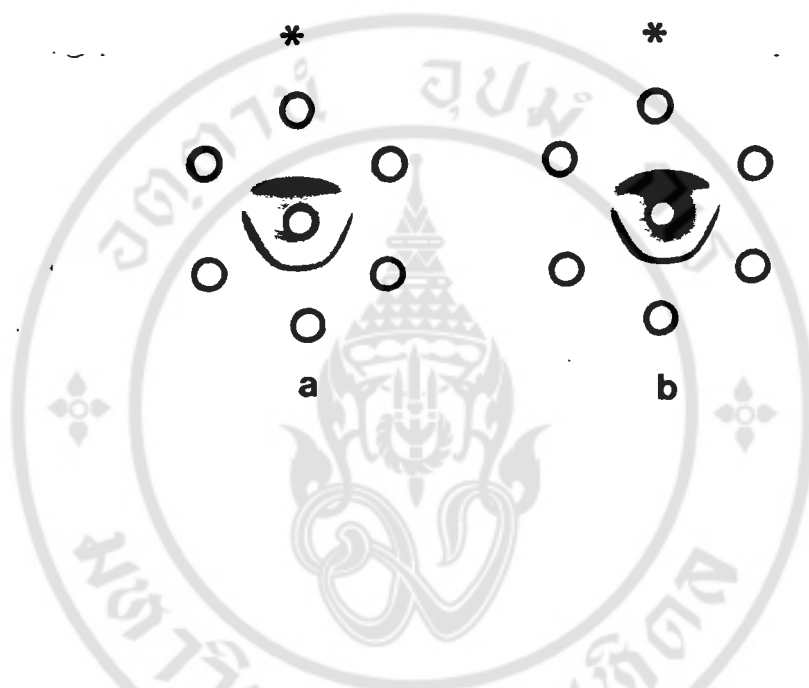


Fig. 18 Immunodiffusion of proteins eluted from Affigel-galactosamine column and GalNAc-Sepharose column. (a) anti-MPL was loaded into center well ; the antigens (starting from asterisk and turn counterclockwise) were : S-60, unbound fraction (Affigel-galactosamine), purified Jack fruit lectin (Affigel-galactosamine), unbound fraction (GalNAc-Sepharose), bound fraction (GalNAc-Sepharose). (b) same as (a) except that 0.03 M GalNAc was mixed with antigens prior to loading into the wells.



Fig. 19 Patterns of sperm agglutination. Sperm agglutination mediated by purified Jack fruit lectin was observed under a phase-contrast microscope. (a) human sperm ; (b) rat cauda epididymal sperm ; (c) rat caput epididymal sperm. The patterns of agglutination were head-to-head ($\blacktriangleleft$), head-to-tail ($\blacktriangleleft$), and tail-to-tail (—).



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4. DISCUSSION

4.1 Survey of plant agglutinating agents

Agglutination of cells occurs when the specific sites on cell membrane of adjacent cells are cross-linked by bifunctional cross-linking agents. The interaction between antibody with two antigens will lead to agglutination of the antigen molecules. The antibody specific to an antigenic determinant on cell surface may act as a bifunctional cross-linking agent leading to cell agglutination when one antibody binds two cells. Lectins which contain at least two sugar-binding sites may also cause cell agglutination. They can bind specifically with carbohydrate moieties of glycoproteins and glycolipids. Since glycoproteins and glycolipids are general components of cell membrane, lectins may then cross-link the adjacent cells. Based on the above cell cross-linking property of lectins, agglutination test is a common method to detect the lectins and to assay lectin activity. Although lectins can agglutinate various types of cells, the cells to be used in the agglutination experiments should be uniform and conveniently available. Usually, red blood cells of mammals, for example human, rabbit, and sheep, are used in agglutination test. Other cells, for example leucocytes, lymphocytes, sperm, and tumor cells can also be used. As cell surface carbohydrates are expected to be different among these cell types, the result of agglutination test may vary from one cell type to another. One of the aims of this study is to look for a new lectin that can agglutinate sperm so that

it can be used as a vaginal contraceptive. Sperm agglutination test has been used as a method to search for lectins in extracts of Thai plants and to assay the lectin activity.

Most lectins previously studied agglutinate human erythrocytes of all blood types with equal activity. However, some lectins are blood type specific, agglutinating preferentially certain blood group erythrocytes. For example, *Dolichos biflorus* lectin and *Ulex europaeus* lectin agglutinate red cells of blood group A₁ and O, respectively (Entlicher et al., 1970). *Vicia gramineae* shows specificity towards blood group N erythrocytes (Prigent et al., 1982). In this study, all of the active seed extracts showed no preference or specificity towards a human blood group (data not shown). They agglutinated red cells from group A, B, and O to the same extent. Some seed extracts (Table 8) have been found to cause hemolysis. The lytic factor in these seed extracts are still unknown. Soybean and peanut seed extracts have been found to agglutinate neuraminidase-treated red cells but not untreated cells, suggesting that removal of sialic acid may expose the "hindered" binding site for agglutinating agents in these two extracts.

In order for a lectin to be useful as vaginal contraceptive, it should be active in the whole semen. For this reason, liquified whole semen sample have been employed in this study. Human seminal plasma normally contains glycoproteins and sugars such as fructose. These components in seminal plasma may compete with human sperm in binding with agglutinating agents. Thus, it will be expected that a higher concentration of protein will be required to cause agglu-

mination of unwashed sperm (whole semen) than needed to agglutinate washed sperm (Table 7). Washing of the sperm to remove the seminal plasma, on the other hand, is essential if one intends to study only the sperm-lectin interaction.

Rat cauda epididymal sperm tend to bind with each other causing some agglutination even in the absence of an agglutinating agent. This situation has not been observed with rat caput epididymal sperm or human sperm. Due to the partial self agglutination, care must be taken in using rat cauda epididymal sperm in the assay of agglutinating activity. A control, rat cauda epididymal sperm in buffer solution, must be performed along side with a seed extract so that an increase in agglutination due to an active extract can be easily observed. Rat sperm agglutination is more sensitive than human sperm agglutination since very low concentration of agglutinating agents is sufficient to cause rat sperm agglutination (Table 7). Human sperm agglutination requires higher amount of agglutinating agents. This implies that rat sperm may contain more binding sites than human sperm. It should be noted that rat sperm is much larger (or longer) than human sperm and hence should have more surface area. Hemagglutination can also detect only the minute amount of agglutinating agents; however, the incubation time is 1 h for hemagglutination but only 1 min for sperm agglutination, so the comparison of the sensitivity between hemagglutination and sperm agglutination is not appropriate.

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Seed extracts from rambutan and longan show different agglutination activities between the two populations of rat sperm, the

caput and cauda epididymal sperm (Table 5). They seem to interact only with caput epididymal sperm suggesting that the sperm may have lost the binding sites for the active agents in rambutan and longan during transition from caput to cauda epididymis. There may be the cleavage, addition or modification of sugar residues to decrease the binding sites during sperm maturation. Reduction of binding site during sperm maturation has also been observed previously for other lectins such as *Ricinus communis* (RCA) and *Ulex europaeus* (UEA) (Hammerstedt et al., 1982). The purified agglutinating agents or lectins from seed extracts of rambutan and longan may be useful in the study of lectin binding sites and surface change of sperm during maturation.

Sa-taw and yard-long bean seed extracts have been found to agglutinate rat sperm and human sperm but not neuraminidase-treated or untreated erythrocytes (Table 9). In contrast, balsam pear seed extract which agglutinates human erythrocytes (Table 9) does not agglutinate rat and human sperm (Table 7). These results clearly demonstrate the principle discussed earlier that the result of the agglutination test can vary with the cell types used. In this study, some lectin sources would have been missed if the screening was done using only one cell type.

The most active seed extracts, namely red kidney bean, broad bean, garden pea, tamarind, Sa-taw, green gram, yard-long bean, and soybean (Table 5) are from plants in family Leguminosae. This result corresponds with the report of Tom and Western (1971) concluding that 95% of lectins so far studied come from the plants in family

Leguminosae. The seeds from other plant families have also been found to contain agglutinating agents. From this studies, agglutinating agents have also been detected in extracts of seeds from family Moraceae (Jack fruit), family Sapindaceae (rambutan, longan), family Gramineae (sorghum, dotted), and family Cucurbitaceae (balsam pear). Apart from these families, lectins have previously been found in plants of family Solanaceae (tomato; Kilpatrick et al., 1983), family Papilionaceae (*Dolichos lablab* ; Guran et al., 1983), family Euphorbiaceae (*Croton tiglium* ; Banerjee and Sen, 1981, *Euphorbia heterophylla* ; Nsimba-Lubaki et al., 1983). Some well-known seed extracts, red kidney bean, broad bean, peanut and soybean, which have been previously shown to contain lectin by using hemagglutination test (Weber et al., 1972; Wang et al., 1974; Lotan et al., 1975; Sharon and Lis, 1972) possess sperm agglutinating activity (Table 7). Whether the active agents for hemagglutination and sperm agglutination is the same cannot be determined at present.

The limited survey of Thai plants conducted in this study has provided new sources of agglutinating agents : Jack fruit, Sa-taw, rambutan, longan, tamarind, horse-tamarind, and balsam pear. Among these plants, Jack fruit appears to contain the most potent agglutinating agent which should be further studied. However, there are many more kinds of Thai plants to be explored. More interesting and new sources of lectins may be awaiting to be discovered.

Since sperm motility is initiated soon after ejaculation, it would be of interest to ask if sperm agglutinating agents can also inhibit the motility initiation. The preliminary results (Table 10)

confirms the expectation that the ability to inhibit initiation of sperm motility corresponds with sperm agglutination activity. Jack fruit seed extracts which contains the most active sperm-agglutinating agent inhibits the initiation of sperm motility completely (Table 10). Other seed extracts showing partial sperm-agglutinating activity also show partial inhibition. However, further study should be performed to compare the potencies among these seed extracts.

4.2 Jack fruit lectin

Affinity chromatography is a common and efficient method to purify lectin (Lis et al., 1974). The choice of the sugar ligand depends on known sugar-binding specificity of a particular lectin. In this case, the finding on the specificity of Jack fruit lectin suggests that N-acetylgalactosamine should be the ligand to be linked with the resin. The reason that the commercially available Affigel galactosamine (Fig. 10) works better than our own coupled product is not entirely clear. It appears that the commercial preparation has a higher capacity than the home-made one.

The purified material from Jack fruit has the necessary properties of a lectin, namely it is a protein sensitive to pronase digestion (Table 15), it binds specifically with N-acetylgalactosamine (Table 12) and it agglutinates cells : rat sperm, human sperm and human erythrocytes (Fig. 2, Fig. 3, and Table 8).

The purified Jack fruit lectin is considered homogeneous as judged from its elution as a single and symmetrical peak from gel filtration on Sephadex G-200 with corresponding symmetrical profiles of hemagglutinating and sperm agglutinating activities (Fig. 11).

Although the proposed trimeric structure of $2 \times 13,000$ and $1 \times 18,000$ best fits the molecular weight of the non-denatured (Fig. 11) and the dissociated forms (Fig. 14, Fig. 15), no attempt has yet been made to prove the structure. Since a way has not been found to dissociate the lectin without using SDS, it is not yet possible to determine which of the subunits carries the sugar-binding sites. Preliminary result (data not shown) suggests no inter-subunit disulphide bond.

In ND-PAGE, the smear protein pattern with the activity spreading over the protein smear and forming two broad activity peaks (Fig. 16, b) suggests that purified Jack fruit lectin may exist in many forms different in charge. The charge heterogeneity may be due to peptide chain modification. Various lectins are glycosylated to different degree and their carbohydrate moieties (if any) may become modified later. Phosphorylation is another common cause of charge heterogeneity of proteins (Vera et al., 1984). In the case of the Jack fruit lectin, the molecular basis of charge heterogeneity has not yet been determined.

The ability of the purified Jack fruit lectin to form a precipitin band with anti-*Maclura pomifera* lectin but not with other antisera tested (Fig. 17) suggests that Jack fruit lectin should have some antigenic site or structure similar to that of *Maclura pomifera* lectin. *Maclura pomifera* lectin has been purified from osage orange seeds which are from the plant of Moraceae family. It is a protein of MW 40,000-43,000 containing two dissimilar subunits of MW 12,000 and 10,000 (Bausch and Poretz, 1977). This lectin has the specificity towards various α -D-galactopyranosides, e.g. galactose and GalNAc.

It contains very small amount of methionine and no cysteine. Thus, it appears that Jack fruit lectin and *Maclura pomifera* lectin coming from plants of the same family may be structurally similar.

There are two possible mechanisms for the antibody-lectin precipitin formation (Fig. 17). One, the antibody recognizes an antigenic site on the Jack fruit lectin and forms complexes. Two, Jack fruit lectin binds sugar moieties of the antibody resulting in the observed precipitin band. The latter possibility has been shown to be incorrect since addition of the specific sugar (GalNAc) does not prevent the precipitin band formation (Fig. 18). Therefore, the antibody should bind to an antigenic site of the lectin molecule and this site is different from sugar-binding sites.

It is uncertain whether Jack fruit seeds contain multiple lectins with different activities in hemagglutination, human sperm agglutination and rat sperm agglutination. These activities seem to be slightly segregated by ammonium sulfate fractionation of the crude Jack fruit extract (Fig. 7). Although the affinity chromatography (Fig. 10) suggests that Jack fruit lectin represents 90% of protein in S-60 fraction, the S-60 fraction only contains about 40 per cent of total soluble protein in the crude seed extract. Therefore, it is uncertain if the agglutinating agent not included in the S-60 fraction will prove to be the same or different from the purified Jack fruit lectin.

The apparent difference between the lectin in the S-60 fraction being resistant to pronase and purified Jack fruit lectin being sensitive to the enzyme (Table 15) should suggest that the

S-60 fraction may contain some protease inhibitor which later become eliminated during the purification of the Jack fruit lectin. The inhibitors to certain proteolytic enzymes have been found throughout the plant kingdom particularly among the legumes (Liener, 1978). The well-known protease inhibitor is trypsin inhibitor isolated from soybean.

Since galactosyl residues are common sugars of glycoproteins and glycolipids, they are widely found on surface of various cells including sperm. So it is not too surprising that Jack fruit lectin having specificity for N-acetylgalactosamine will cause random patterns of agglutination of sperm (Fig. 19).

Although this study has successfully identified Jack fruit as a new source of a new lectin, the sugar-specificity of the Jack fruit lectin (for galNAc) is unfortunately shared by other known lectins such as soybean lectin (Gordon et al., 1972), *Phaseolus limensis* lectin (Galbraith and Goldstein, 1972), *Phaseolus vulgaris* lectin (Weber et al., 1972), *Sophora japonica* lectin (Terao and Osawa, 1973; Poretz et al., 1974), *Wistaria floribunda* lectin (Toyoshima et al., 1971), *Dolichos biflorus* lectin (Pere et al., 1974). It is, therefore, not yet possible to predict the advantage Jack fruit lectin will have over these other lectins in term of future use in cell surface study or as vaginal contraceptive.

5. SUMMARY

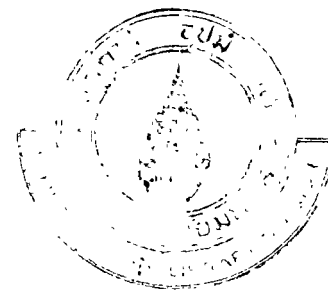
1. Among thirty-one kinds of seeds, one kind of storage roots, and one kind of stem tested, fifteen kinds of seeds contained agglutinating activity. Most of the active seed extracts were from the plants in family Leguminosae (red kidney bean, broad bean, garden pea, tamarind, horse tamarind, Sa-taw, green gram, peanut, yard-long bean, and soybean). Other active seed extracts were from the plants in family Moraceae (Jack fruit), family Sapindaceae (rambutan, longan), family Gramineae (sorghum, dotted), and family Cucurbitaceae (balsam pear).
2. Some seed extracts showed positive agglutination test on all four cell types used, namely red blood cells, human sperm, rat caput and cauda epididymal sperm. However, some extracts only gave positive test with certain but not all cell types.
3. Jack fruit extract was found to completely inhibit the initiation of sperm motility while extracts from red kidney bean and garden pea also showed strong inhibitory effect.
4. Jack fruit lectin was purified by ammonium sulfate precipitation, followed by affinity chromatography on an Affigel-galactosamine column.
5. The purified Jack fruit lectin was sensitive to pronase and was specific to GalNAc. It was eluted as a single and symmetrical peak from a column of Sephadex G-200 with a molecular weight of 42,000. It composed of two dissimilar subunits showing molecular weight of 18,000 and 13,000 on SDS-PAGE. A trimeric structure

consisting of two small and one large subunits was proposed.

Electrophoresis of the purified material under non-denaturing condition showed smear patterns of protein and activity, possibly indicating multiple forms of lectin carrying different charges.

6. By immunodiffusion (Ouchterlony test), Jack fruit lectin was shown to form one precipitin band with anti-*Maclura pomifera* lectin derived from another plant of the same family.





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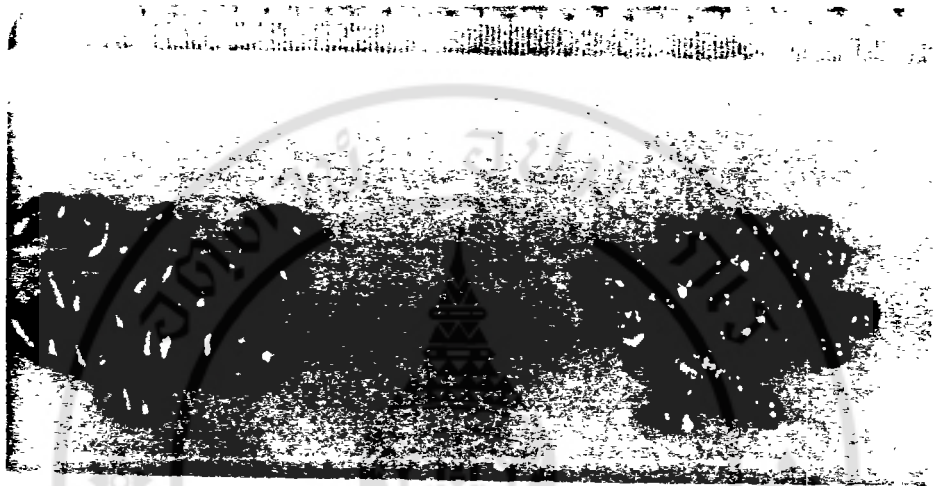
Appendix

1. List of Thai plants used in this study.

<u>Family</u>	<u>Common name</u>	<u>Thai name</u>	<u>Scientific name</u>
Annonaceae	Custard apple	น้อยหน่า	<i>Annona squamosa</i>
Bombacaceae	Durian	ทุเรียน	<i>Durio zibethinus</i>
Caricaceae	Papaya	มะละกอ	<i>Carica papaya</i>
Compositae	Sunflower	ทานตะวัน	<i>Helianthus annuus</i>
Cucurbitaceae	Balsam pear	มะระ	<i>Momordica charantia</i>
Euphorbiaceae	Cassava	มันสำปะหลัง	<i>Manihot esculenta</i>
Gramineae	Corn	ข้าวโพด	<i>Zea mays</i>
	Job's tear	เคียว	<i>Coix lachryma-jobi</i>
	Rice	ข้าว	<i>Oryza sativa</i>
	Sorghum (dotted)	ข้าวฟ่างลาย	<i>Sorghum bicolor</i>
	Sorghum (red)	ข้าวฟ่างแดง	<i>Sorghum vulgare</i>
Leguminosae	Black gram	ถั่วดำ	<i>Vigna mungo</i>
	Broad bean	ถั่วปากอ้า	<i>Vicia faba</i>

<u>Family</u>	<u>Common name</u>	<u>Thai name</u>	<u>Scientific name</u>
Leguminosae	Garden pea	ถั่วลันเตา	<i>Pisum sativum</i>
	Green gram	ถั่วเขียว	<i>Vigna radiata</i>
	Horse-tamarind	กระถิน	<i>Leucaena leucocephala de Wit</i>
	Lentil	ถั่วแดง	<i>Lens esculenta</i>
	Peanut	ถั่วลิสง	<i>Arachis hypogaea</i>
	Red kidney bean	ถั่วแดงหลวง	<i>Phaseolus vulgaris</i>
	Sa-taw	สะตอ	<i>Parkia speciosa</i>
	Soybean	ถั่วเหลือง	<i>Glycine max</i>
	Tamarind	มะขาม	<i>Tamarindus indica</i>
	Winged bean	ถั่วพู	<i>Psophocarpus tetragonolobus</i>
	Yard-long bean	ถั่วฝักยาว	<i>Vigna unguiculata</i>
Malvaceae	Jamaica sorrel	กระเจี๊ยบ	<i>Hibiscus sabdariffa</i>
Moraceae	Jack fruit	ขี้หนุ	<i>Artocarpus heterophyllus</i>
Nymphaeaceae	Lotus	บัว	<i>Nelumbo nucifera</i>
Palmae	Salacca	ระกำ	<i>Salacca wallichiana</i>

<u>Family</u>	<u>Common name</u>	<u>Thai name</u>	<u>Scientific name</u>
Sapindaceae	Litchi	ลิ้นจี่	<i>Litchi chinensis</i>
	Longan	ลำไย	<i>Dimocarpus longan</i>
	Rambutan	เงาะ	<i>Nephelium lappaceum</i>
Sapotaceae	Sapodilla	ละมุด	<i>Achras sapota</i>
Solanaceae	Potato	มันฝรั่ง	<i>Solanum tuberosum</i>



น้อยหน้า

CUSTARD APPLE
Annona squamosa

มะละกอ

PAPAYA
Carica papaya



กระถิน

HORSE TAMARIND
Leucaena leucocephala de Wit

ทุเรียน

DURIAN
Durio zibethinus



มะขาม
TAMARIND
Tamarindus indica

ถั่วดำ
BLACK GRAM
Vigna mungo



ถั่วแดงหลวง
RED KIDNEY BEAN
Phaseolus vulgaris

สะตอ
SATAW
Parkia speciosa



ถั่วแดง
LENTIL
Lens esculenta

กระเจียบแดง
JAMAICA SORREL
Hibiscus sabdariffa



ขนุน
JACK FRUIT
Artocarpus heterophyllus

ถั่วเขียว
GREEN GRAM
Vigna radiata



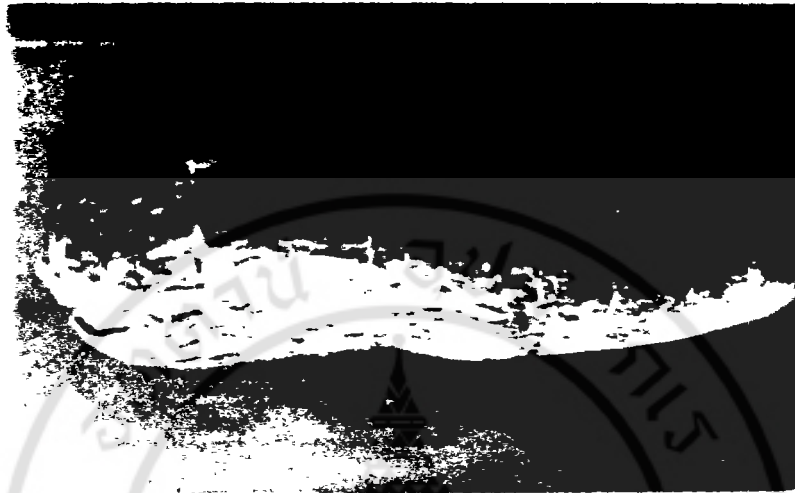
ถั่วเหลือง
SOYBEAN
Glycine max

ถั่วลิสง
PEANUT
Arachis hypogaea



ถั่วลันเตา
GARDEN PEA
Pisum sativum

ถั่วฝักยาว
YARD LONG BEAN
Vigna unguiculata



มันสำปะหลัง

CASSAVA

Manihot esculenta



เงาะ

RAMBUTAN

Nephelium lappaceum

ทานตะวัน

SUNFLOWER

Helianthus annuus

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มะระ

BALSAM PEAR

Momodica charantia

ถั่วปีก

WINGED BEAN

Euphycomia carinata



ถั่วปากอ้า

BROAD BEAN

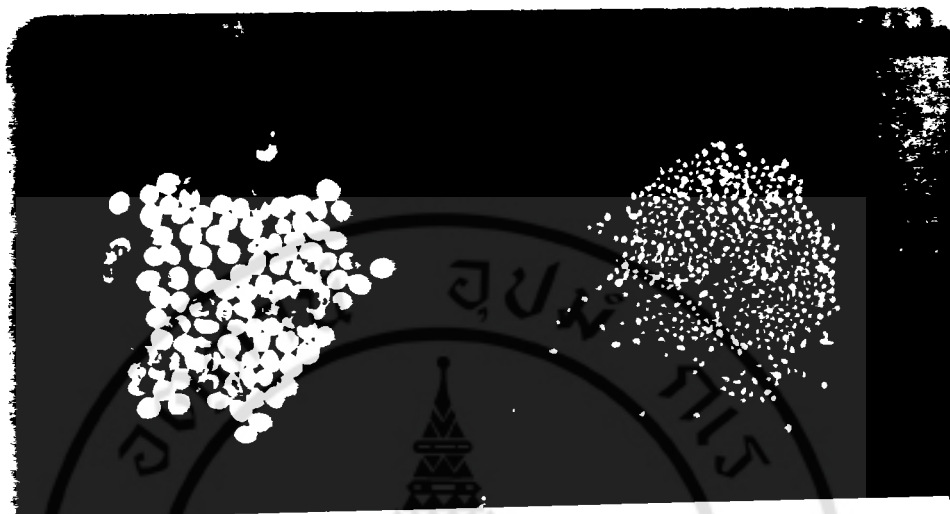
Vicia faba

มันฝรั่ง

POTATO

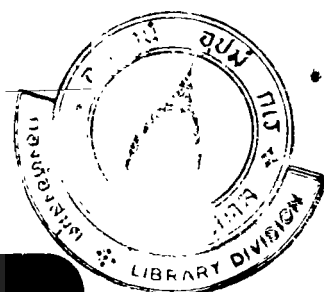
Solanum tuberosum

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ข้าวฟ่างลาย
SORGHUM (dotted)
Sorghum bicolor

ข้าวฟ่างแดง
SORGHUM (red)
Sorghum vulgare



เดือย
JOB'S TEAR
Coix lachyma-jabi

ข้าวโพด
CORN
Zea mays



ข้าว
RICE
Oriza sativa

ลำไย
LONGAN
Dimocarpus longan



ลิ้นจี่
LITCHI
Litchi chinensis

ละมุด
SAPODILLA PLUM
Manikara achras



บัว
LOTUS
Nelumbo speciosa

ระกำ
SALACCA
Salacca wallichiana

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