Purification and Characterization of *Hevea* latex lectin and Its Binding Protein from Latex of *Hevea brasiliensis*

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Thesis Title	Purification and Characterization of *Hevea* latex lectin and Its Binding Protein from Latex of *Hevea brasiliensis*
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ชื่อวิทยานิพนธ์	การทำให้บริสุทธิ์และศึกษาคุณสมบัติของเลคตินและโปรตีนที่เกาะจับกับเลคตินในน้ำยางพารา
ผู้เขียน	นางสาวปิยาภรณ์ ภาษิตกุล
สาขาวิชา	ชีวเคมี
ปีการศึกษา	2543

บทคัดย่อ

ได้ค้นพบว่า โปรตีนชนิดหนึ่งซึ่งเตรียมได้จากส่วนของ bottom fraction (BF) ที่แยกได้หลังจากการปั่นน้ำยางสด มีคุณสมบัติเป็นเลคติน คือ สามารถเหนี่ยวนำให้เม็ดเลือดแดงของกระต่ายและหนูให้เกาะกลุ่มได้แต่ไม่สามารถเหนี่ยวนำเม็ดเลือดแดงของคนได้ โปรตีนนี้ให้ชื่อว่า *Hevea* latex lectin (HLL) และสามารถเตรียมได้จากการนำ BF มาตกตะกอนด้วยอะซีโตน แล้วทำการสกัดต่อด้วย 0.2% Triton X-100 ซึ่งเป็น non-ionic detergent จากนั้นนำสารสกัดที่ได้ไปทำให้บริสุทธิ์ต่อด้วย chitin batch-binding และ แยกผ่าน คอลัมน์ DEAE-Sepharose จะได้ HLL ที่บริสุทธิ์มีน้ำหนักโมเลกุลย่อย 17 กิโลดาลตัน โดยวิธี SDS-PAGE และมีน้ำหนักโมเลกุลรวม 412 กิโลดาลตัน โดยวิธี gel filtration HLL ที่ได้นี้มีคุณสมบัติทนต่อสภาวะด่างได้ดี แต่ไม่ทนในสภาวะกรด ที่มีค่า pH ต่ำกว่า 5 นอกจากนี้ยังทนต่อ pronase และ ความร้อนได้เป็นอย่างดี โดยพบว่า แอคติวิตี้จะคงเหลืออยู่ครึ่งหนึ่งหลังจากการอุ่นที่อุณหภูมิ 70-80 องศาเซลเซียส ค่า pI ของ HLL เท่ากับ 7.2 ซึ่งจัดเป็น neutral โปรตีน นอกจากนี้ยังพบว่า HLL ที่ได้จากการทำบริสุทธิ์มีความสามารถในการเหนี่ยวนำการเกาะกลุ่มของอนุภาคยาง

ความสามารถในการเหนี่ยวนำเม็ดเลือดแดงของ HLL พบว่าถูกยับยั้งได้โดยไกลโค-

โปรตีนหลายชนิด ทั้งที่มาจากแหล่งน้ำยางเอง และจากแหล่งอื่นๆ สำหรับน้ำยางแหล่งที่พบไกลโคโปรตีนยับยั้ง ได้แก่ ส่วนของ C-serum และ rubber layer ที่ได้หลังจากการปั่นแยกน้ำยางสดด้วยเครื่อง ultracentrifuge ส่วนไกลโคโปรตีนยับยั้งที่พบจากแหล่งอื่นๆ มีหลายชนิด ได้แก่ fetuin, asialofetuin, ovomucoid, mucin, asialomucin และ type II-S trypsin inhibitor โดยพบว่า HLL จะมีความสามารถในการเกาะจับจำเพาะกับ ไกลโคโปรตีนที่มาจากแหล่ง น้ำยาง ได้สูงกว่า แหล่งอื่นๆ และ มีความจำเพาะในการเกาะจับกับ ไกลโคโปรตีนที่มาจาก C-serum ได้ดีกว่าส่วนที่มาจากอนุภาคยาง ในบรรดาไกลโคโปรตีนที่มาจากแหล่งอื่นๆ พบว่า HLL จะมีความจำเพาะ ในการเกาะจับกับไกลโคโปรตีนที่มีองค์ประกอบของ sialic acid ได้ดีกว่าไกลโคโปรตีนที่ปราศจาก sialic acid ซึ่งในการทดลองได้ทำการเตรียมโดย การย่อย bovine submaxially mucin ด้วย neuraminidase นอกจากนั้น ยังพบว่า น้ำตาลอิสระ หลายชนิดจำพวก mono-, di หรือ tri- ไม่สามารถยับยั้งแอคติวิตี้ของ HLL ได้

การทำบริสุทธิ์ C-serum HLL binding protein (CS-HLLBP) จากส่วนของ C-serum สามารถทำโดยการตกตะกอนโปรตีนแบบต่อเนื่องด้วยเกลือแอมโมเนียม แล้วตามด้วยอะซีโตน จากนั้นนำสารสกัดที่ได้มาทำบริสุทธิ์ต่อด้วยการผ่านคอลัมน์ gel filtration และ ion exchange จากผลการวิเคราะห์ พบว่า CS-HLLBP ที่ทำบริสุทธิ์ได้จะมีน้ำหนักโมเลกุลย่อย 40 กิโลดาลตัน โดยวิธี SDS-PAGE และมีน้ำหนักโมเลกุลรวม 240 กิโลดาลตัน โดยวิธี gel filtration CS-HLLBP ที่ได้สามารถทนต่อความร้อนได้สูงถึง 50 องศาเซลเซียส มีค่า pI เท่ากับ 4.7 และทนต่อ pH ได้ดีในช่วง 6-10 แอคติวิตี้ของ CS-HLLBP จะลดลงเมื่อถูกย่อยด้วย chitinase นอกจากนี้ยังพบว่า CS-HLLBP สามารถออกฤทธิ์ยับยั้งการเกาะกลุ่มของอนุภาค

ยางที่เกิดขึ้นโดยการเหนี่ยวนำของ HLL และยังพบว่าค่าสัมประสิทธิ์ความสัมพันธ์ระหว่างปริมาณ CS-HLLBP กับปริมาณน้ำยางสดต่อครั้งการกรีดสูงถึง 0.97

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Abstract

A protein in bottom fraction (BF) of centrifuged fresh latex was found to possess lectin activity by being able to agglutinate erythrocytes from rabbits and mice but not human. The *Hevea* latex lectin (HLL) was prepared from the BF by acetone precipitation and follow by extraction in the presence of a non-ionic detergent, 0.2% Triton X-100. It was purified to homogeneity by employing chitin batch-binding and DEAE-Sepharose column. Its M_r upon SDS-PAGE is 17 kD with native M_r obtained by gel filtration of 412 kD. It is stable under strong alkalinity and acid labile when pH < 5. It is resistant to pronase digestion and relatively heat stable where approximately half of total activity was retained at 70-80 ^{O}C. It is a neutral protein with a pI value of 7.2. The purified HLL was found to be able to induce aggregate formation among small rubber particles (SRP).

Several glycoproteins from both latex and non-latex origin were found to be able to inhibit hemagglutination induced by HLL. The HLL inhibitors in latex were found in C-serum and rubber particle fractions of centrifuged fresh latex. For the non-latex glycoproteins, fetuin, asialofetuin,

ovomucoid, mucin, asialomucin, type II-S trypsin inhibitor were found to be HLL inhibitor. The HLL-binding specificity was higher with proteins originated from latex than any other sources. Moreover, its binding is more effective with latex protein purified from C-serum than rubber particle. For the non-latex glycoproteins, the protein containing sialic acid was shown to be more effective in binding with HLL. A decrease in hemagglutination inhibition level was observed when sialic acid was remove from a bovine submaxially mucin under neuraminidase treatment. Various free sugars, mono-,di-, or tri-saccharides failed to inhibit hemagglutination induced by HLL.

The C-serum HLL binding protein (CS-HLLBP) was purified from the C-serum fraction by subjected to ammonium sulfate fractionation, acetone precipitation, gel filtration and ion-exchange column chromatography. Purified CS-HLLBP possessed M_r of *ca* 40 kD upon SDS-PAGE. The native M_r after gel filtration was *ca* 240 kD. It is heat stable upto 50 °C. The pI value was around 4.7 while a broad range of pH stability was observed from 6-10. The ability of CS-HLLBP in inhibiting hemagglutination activity of HLL was found to be reduced upon prior chitinase treatment. The purified CS-HLLBP, without any prior chitinase-treatment, was able to inhibit HLL-induced SRP aggregate formation. In addition, a highly positive correlation value ($r = 0.97$) was obtained between levels of latex CS-HLLBP and rubber yield per tapping.

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Piyaporn Pasitkul

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List of Abbreviations

^0C	=	degree celsius
BSA	=	bovine serum albumin
EDTA	=	ethylenediamine tetraacetic acid
TEMED	=	N,N,N',N'-tetramethyl ethylenediamine
PAGE	=	polyacrylamide gel electrophoresis
SDS	=	sodium dodecyl sulfate
mg	=	miligram
µg	=	micogram
nm	=	nanometer
g	=	gravitation acceleration
h	=	hour
M	=	molar
mM	=	millimolar
ml	=	milliliter
µl	=	microliter
min	=	minute
OD	=	optical density
%	=	percentage
sec	=	second
MW, M_r	=	molecular weight

U	=	unit
R_f	=	relative mobility
kD	=	kilodalton
H.A.	=	hemagglutination activity
H.I.	=	hemagglutination inhibition activity
Tris	=	Tris(hydroxymethyl)aminomethane
GlcNAc	=	N-acetyl-D-glucosamine
GalNAc	=	N-acetyl-D-galactosamine
Xyl	=	xylose
Man	=	mannose
Fuc	=	fucose
Raf	=	raffinose
Glc	=	glucose
Gal	=	galactose
Ara	=	arabinose
HLL	=	*Hevea* latex lectin
CS-HLLBP	=	C-serum *Hevea* latex lectin binding protein
SRP-HLLBP	=	small rubber particles *Hevea* latex lectin binding protein

Chapter 1

Introduction

Hevea brasiliensis is today the main source of natural rubber in the world. This tree has a complex laticiferous system consisting of anastomosed cells arranged in monocellular layers around the cambium (Dickenson, 1964; Hebant and de Faÿ, 1980). These cells have special features ; in addition to the classic element a great number of particles of rubber, plastid type structures : Frey-Wyssling particles, and lysosomal microvacuoles : lutoids form a real cell compartment whose relationship with the cytosol plays an extremely important biological role (d' Auzac *et al.*, 1982).

Nowadays, Thailand as a major rubber latex producer has expanded planting areas to all over the country to increase latex production to meet demand of the world market. Increasing latex production of *Hevea* has always been a major objective for planters. Because rubber tree has long life span, selection of high-yield seedling of rubber tree before planting is important and economically sound. However, a better and effective way to prevent the planting of low-yield rubber trees is still under investigation for practical uses and development.

The study on biochemical markers for selection of high-yield seedling have been investigated. There are two markers involved for the approach. The first the marker is concerning with rubber biosynthesis capacity pathway. This marker has been targeted on the enzyme controlling rubber biosynthesis rate which can serve as enzyme marker for high yield seedling. It was found that a rate-limiting enzyme in rubber biosynthesis pathway, β-hydroxy-β-methylglutaryl CoA reductase (HMGR), has positive correlation with the latex yield (Wititsuwannakul, 1990a). This enzyme can probably be used as a marker for selecting high-yield rubber seedling by criteria of high rubber biosynthesis capacity.

The second marker related to the flow duration process of latex after tapping. Longer flow duration will usually result in the higher rubber yield. The progressive plugging of latex vessels during the course of latex flow following tapping restricts the quanlity of latex being exuded and is hence an important determinant of yield output. Various hypothesis have been advanced to explain the mechanisim of latex vessel plugging (Southorn, 1969), many of them emphasising an involvement of damage to the lutoid (Southorn, 1968). Thus, the study on the biochemical process in latex vessel plugging of *Hevea brasiliensis* may be divided into two co-operative processes. One is an enzymatic dependent leading to lutoid bursting and

another is a non-enzymatic process involving coagulum formation to induce plugging.

For this work, we focused our study on the non-enzymatic process, especially on the specific binding among proteins involved in coagulum formation.

Literature Review

1. Rubber latex

1.1 Rubber latex vessels

The latex vessels of *Hevea brasiliensis* occur in almost all parts of the tree. Althought the principal latex-bearing tissue is the secondary phloem of the trunk, phloem associated with the roots, branches and leaves also have latex vessels. They are present in the flowers and fruits (even in the inner integuments of the seed), the cotyledons and in the pericarp of the fruit. *Hevea* latex vessels are of the articulated anastomosing type. In the secondary phloem, they arise from longitudinal rows of cells from their cambial initials and are laid down at regular intervals to form concentric rings of latex vessels in the trunk (Gomez, 1975). The vessels within a ring are connected frequently by anastomoses and there are few or no connections between rings .

From ontogenic and functional criteria the following types of latex vessels may be distinguished (Gomez, 1975):

Type a. Embryonic vessels in the primary phloem

Type b. Young vessels in the secondary phloem of the tender parts of the tree

Type c. Young vessels in the secondary phloem from mature parts of the tree which escape the tapping operation

Type d. Mature vessels from the trunk which are regularly tapped

Type e. Senescent vessels in the outer bark.

Latex vessels in *Hevea brasiliensis* exhibit differences in cytological complexity depending on the maturity of the vessels and their places of origin. Thus, primordial vessels in the primary phloem exhibit the full cytological machinery present in ordinary parenchyma cells with some specialised features like the existence of rubber particles and lutoids. The occurrence of proplastids and the absence of Frey-Wyssling complexes are features of early specialisation.

Two types of young laticifers in the secondary phleom have been distinguished those in tender parts of the tree (Type b) Fig. 1 and those near the cambium (Type c) in the physiologically more mature region of the tree-the brown bark. Although Type b and c vessels are both juvenile, in a sence, the latter lack some of the juvenile features of the former. The presence of the protein microfibrils in the lutoids is very characteristic of Type b vessels but they have not been seen in the lutoid of Type c vessels. The presence of mitochondria, endoplasmic reticulum, nuclei and ribosomes has been demonstrated for both type of vessels. The Frey-Wyssling complex is also present in both types: this organelle has highly unusual features, but the double membrane suggests that it is a plastid. The Frey-Wyssling complexes are apparently at an intermediate stage of development, since they do not show all the special features of the mature organelle.

The vessels in regular tapping (Type d) contain rubber particles, lutoids, Frey-Wyssling complexes, mitochondria and nuclei, although the nuclei are only seen rarely (Fig. 2).

The tapped latex, lutoid and Frey-Wyssling complexes are numerous. Mitochondria and nuclei have been found in tapped latex but are rare; ribosome can be easily demonstrated in tapped latex after ultracentrifugation.

In extremely senescent vessels the cytological machinery is obscured by the high packing density of rubber particles. It seems likely that the cytoplasmic organelles have in fact degenerated in such vessels, as suggested by Dickenson (1969).

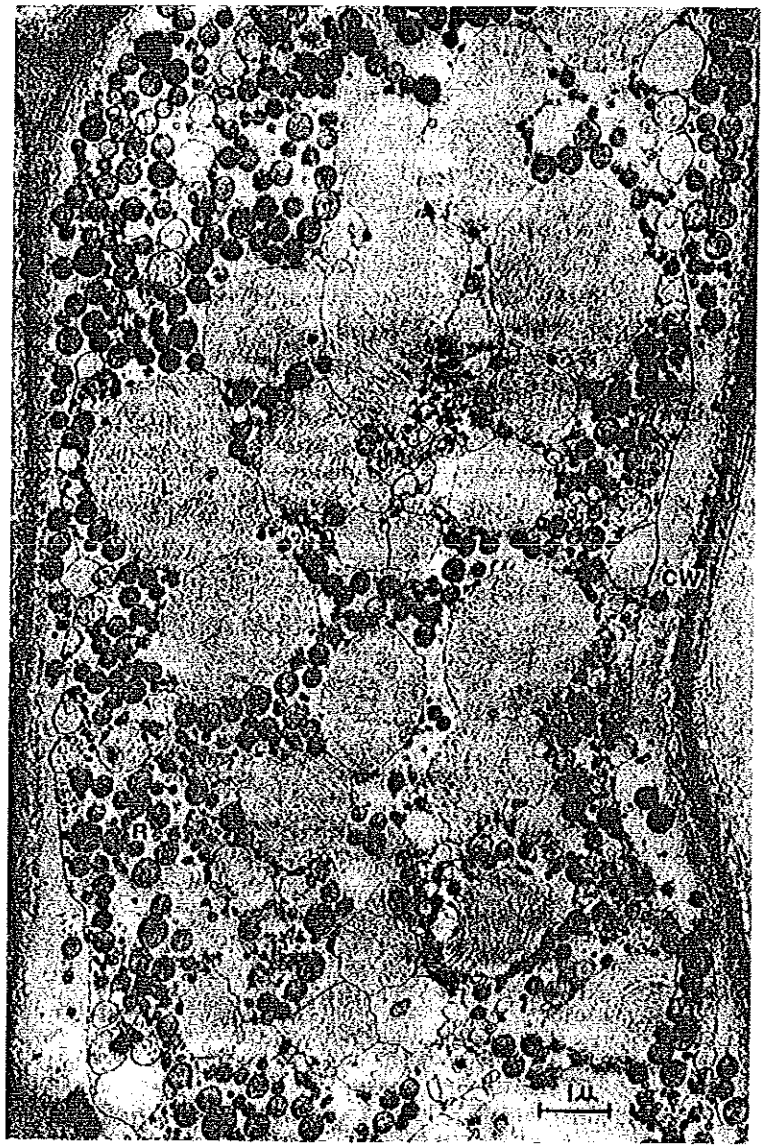


Fig. 1 Young latex vessel (Type b) from the secondary phloem of tender stem showing cell wall (CW), lutoid (L), and rubber particle (R) (x1,100) (Gomez, 1975).

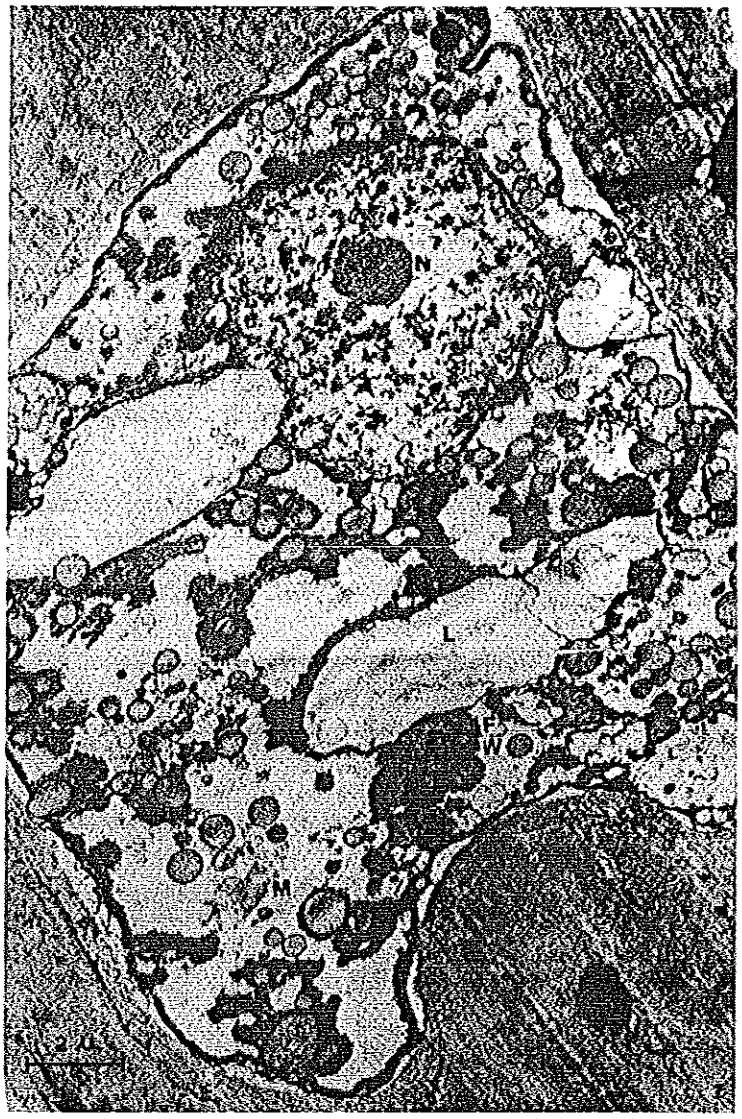


Fig. 2 Cross-section of mature latex vessel (Type d) in regular tapping showing Frey-Wyssling complex (FW), lutoid (L), mitochondria (M) and nucleus (N) (x7,500) (Gomez, 1975)

1.2 Rubber latex composition and fractionation

Rubber is found in more than 2,000 plant species, but is limited to only a few families (Archer *et al.*, 1963a). The genus *Hevea* is a member of the family Euphorbioaceae and comprises several species. Among them, *Hevea brasiliensis* which grows in the hot humid intertropical regions, is the best rubber producer. The latex of this rubber tree species, is collected by tapping the bark tissues. It is a parennial rubber tree with a straight trunk covers with fairly smooth, whitish-gray bark surface. Native rubber trees can grow as high as 40 m with a life span of almost 100 years. However, nowadays the height of rubber trees in most plantations rarely exceed 25 m because its growth is retarded by tapping and they are usually being replanted every 25-35 years after the declining yield reaches an uneconomic level (Webster and Paardekooper, 1989)

Fresh latex from *Hevea brasiliensis* is a specialised cytoplasm containing a suspension of rubber particles and other organelles in an aqueous serum (the cytosol). The latex collected by regular tapping consists of the cytoplasm expelled from the latex vessels. Apart from water, it contains about 30-40 per cent of rubber and about 3.5 per cent of other substances. The structure and composition of fresh, tapped latex was firstly elucidated by high-speed centrifugation (Fig.3) (Mori, 1959). Depending on the centrifugation employed method but in general major zones can clearly be distinguished. The top fraction consists almost entirely of latex rubber; the middle zone are

made up of the watery phase of the latex, generally called C-serum; the relatively heavy bottom fraction, normally yellowish, viscid and semi-liquid, consists mainly of the lutoids. The yellow, lipid-containing Frey-Wyssling complexes are normally found beneath the upper border rubber layer fraction.

Rubber particles

The rubber particles fraction obtained by high-speed centrifugation contains rubber hydrocarbon covered by rubber particle membranes associated the proteins and phospholipid. The rubber hydrocarbon is cis-polyisoprene (Archer *et al.*, 1982) which has a molecular weight of 10^5- 10^7, consists of long chain of the monomer isoprene (C_5H_8). The rubber particles in young trees are spherical in shape, but in mature trees the larger ones may be pear-shaped (Gomez and Moir, 1979). The size of rubber particles usually range from 5 nm to 3 μm and each is surrounded by a surface film (approximately 10 nm thick) composed of proteins, glycoproteins and lipids. These lipids include phospholipids, triglycerides, sterols and tocotrienols (Ho *et al.*, 1975). The film which surrounds rubber particles gives them a negative charge shown by a surface potential (zeta potential). This film carries a negative charge and is responsible for the stability of the rubber particles suspended in the latex serum (Southorn, 1969).

The rubber particles fraction can be devided into 3 distinct zones (Moir, 1959). Zone 1, 2 and 3 contain the rubber particles of different sizes. The biggest rubber particle size is found in zone 1 (Southorn, 1991) . The size of the particle in zone 2 varies from 0.05 to 0.25 μm, these particles are frequently elliptical and sometimes connected by fragments of membrane that might be endoplasmic reticulum. The particles in zone 3 are of lower average size (0.035 to 0.2 μm) and often appear to be linked to each other. The difference in location of these rubber particles in zones 1, 2 and 3 after centrifugation was due to differences in molecular weight of rubber as well as protein contents (Hamzah and Gomez, 1982).

C-serum

The serum phase of the latex (C-serum) contains most of the soluble substances normally found in plant cells (inositols, carbohydrates, free amino acids, proteins, iorganic anions and metal ions). Several different enzymes and intermediates of various metabolics biochemical processes are present in the C-serum. It was actively involved in rubber biosynthesis. The enzyme prenyl transferases for polyprenol biosynthesis and rubber elongation are well documented (Bernard, 1965). Calmodulin in the C-serum (Wititsuwannakul, 1990b) was reported to activate the rate-limiting enzyme, β-hydroxyl-β- methylglutaryl CoA reductase (HMGR), in rubber biosynthesis (Wititsuwannakul, 1990a)

Lutiod particles

The lutoids in *Hevea* latex was first discovered by Homans *et al.*,(1948). They were reported to have a considerable influence on the latex flow characteristics (Southorn and Edwin, 1968) and possess properties of vacuoles (Dickenson, 1964; Gomez and Moir, 1979) or lysosomes (Pujarniscle, 1968). Interesting electron microscopy ultrastructural features had been described by in lutoids from latex vessels in young tissue by Dickenson (1965) and Archer *et al.*, (1963). They observed bundles of tightly coiled helical structures of microfibrils which were subsequently isolated and characterised as a single protein by Audley (Audley, 1965; Audley, 1966; Audley and Cockbain, 1966). This microfibrillar protein was absent from lutoids of mature tissues.

In centrifuged fresh latices, the bottom fraction consisting mainly of lutoid particles with numerous other co-sedimenting particles occupy volumes of 18.5-36% of total latex (Resing, 1995). The lutoid particles are specialized membrane bound organelles found in latex. They are fragile and are osmotically sensitive (Ruinen, 1956). In size they vary from 0.5-3 μm and bounded by a unit membrane about 80 A° thick (Dickenson, 1965; Gomez and Moir, 1979). Repeated freezing and thawing of the bottom fraction resulted in lutoid membrane rupture and their liquid content, often referred to as B serum, could be expelled. The B-serum is

acidic in pH with a value 5.5, its contents included divalent cations (Mg^{2+} and Ca^{2+}) and positively changed proteins which can neutralize the negative charges of rubber particles (Southorn and Edwin, 1968). The B-serum was demostrated to be capable of flocculating rubber particles. The integrity of lutoids was suggested to therefore play an important part in the stability and flow of the latex (Southorn, 1969)

Frey-Wyssling particles

The Frey-Wyssling particles are mainly composed of lipid material and is yellow or orange in color due to the presence of carotenoids. These particles commonly occurred in clusters of two or three (Frey-Wyssling, 1929). The clusters are enclosed in membrane (Southorn, 1962) and are part of a large structure called the Frey-Wyssling complex (Dickenson, 1969). This comprises a spherical vesicle of 4-6 μm in diameter. It is easily deformed but stronger and less osmotically sensitive than the lutoids. It is bounded by a double membrane and containing several Frey-Wyssling particles together with other structures. The latter include a group of tubules embedded in a membrane bound matrix and associated with 2-4 concentric lamellae formed from double unit membrane.

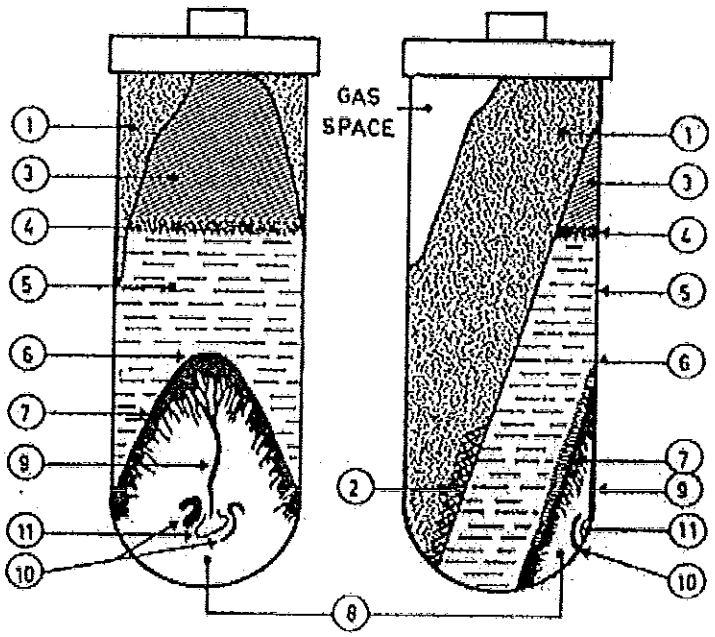


Fig. 3 Separation of fresh latex by ultracentrifugation (53,620 g x 40 min). Fraction 1-3 correspond to white rubber phase. Fraction 4 is a yellow orange layer constituted by Frey-Wyssling particles. Fraction 5 is an almost clear serum (C-serum) corresponding to the latex cytosol. Fraction 6 to 11 constitute the "bottom fraction" in which highest in quantity is the lutoid fraction (Moir, 1959).

1.2 Latex tapping

Latex tapping involved careful stripping of inner bark of rubber tree without damaging the adjacent cambium tissue. The relatively high turgor pressure level in this tissue causes the expulsion of the contents of the lactiferous vessels (Southorn, 1969) with the exception of the nuclei and the mitochondria which have a parietal intracellular location. The latex thus collected can be considered to be a true cytoplasm (Dickenson, 1965)

Before tapping, latex is retained in the vessels at a relatively high hydrostatic pressure, usually between 10 and 15 atm in the early morning. Immediately after tapping, the pressure at the cut ends of the vessels is reduced to ambient. The elastic contraction of the vessel walls under the presure of the still turgid surroundings expels latex at high speed. It had been shown that the turgor pressure of the laticiferous system beneath the cut falls considerably and very rapidly, but the pressure becoming declined steadily less with greater distance apart from the cut (Buttery and Boatman 1967). After tapping, the rate of latex flow can be expected to slow down with time because latex being withdrawn over greater distances from the cut. After the early stages of fast flow, the vessel constriction was found to be insufficient to impede or even stop the flow since its diameter was shown to reduce no more than 25 per cent, even at the position closed to the cut where the constriction was greatest (Pyke, 1941; Gooding, 1952 and Boatman, 1966).

1.3 Latex vessel plugging

A major advance in understanding the mechanism of latex flow resulted form the work of Boatman (1966) and Buttery and Boatman (1966 and 1967). Their experiments involved reopening the tapping cut by bark stripping approximately 1 mm thick at every 10 min intervals and showed that flow rates recovered markedly after each reopening of the vessel ends. This indicated that the flow rate is markedly decreased within a very short time right after tapping by some impediment developing at, or within about 1 mm of, the cut ends of the latex vessels. The nature of the impediment was studied under optical and electron microscope on the longitudinal sections of latex vessels near the tapping cut obtained before and after flow cessation (Southorn, 1968). For the sections made before flow cessation internal plugs of rubber particles and damaged lutoids were observed to completely block some of the vessels. This plug formation was suggested to slowly initiate at the walls of the vessels and grow inwards. In sections made after flow cessation, there was, in addition to the internal plug, also an external cap of coagulum (Fig. 4). This cap had the same composition as the internal plugs which sometimes appeared to have invaded the vessels just before flow ceased (Buttery and Boatman, 1967). Hence, the mechanism of latex vessel plugging in tapped rubber trees resulted in minimizing the loss of its latex metabolite products and involved in inducing plug formation to seal up the wounded latex vessels. It was found that the on-set for wound response in rubber

particles coagulation and latex vessel plugging varied among different rubber trees and clones.

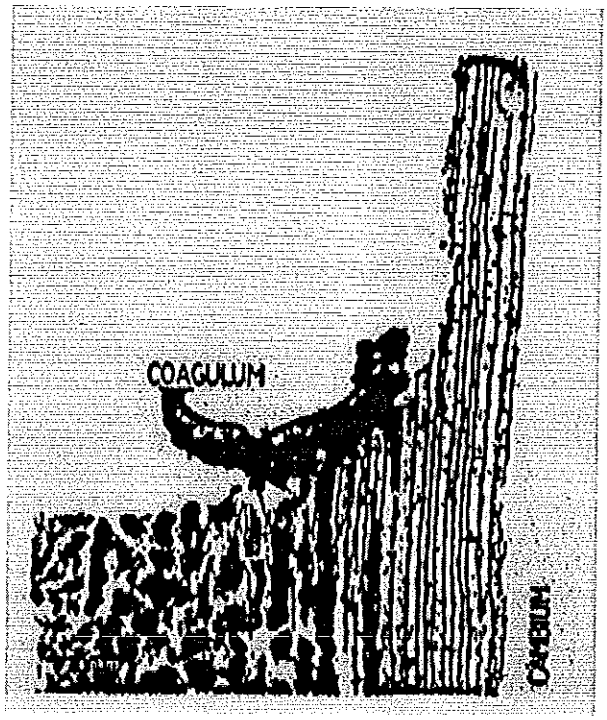


Fig. 4 Radial section from mosaic of optical sections giving overall visualisation of tapping cut (Southorn, 1968)

1.4 Factor involved in lutoid bursting upon tapping

The natural coagulation begins by the appearance of microflocs of degraded lutoid and rubber particles. The lutoid content was one of the major element avaiable in the laticiferous vessels during the flow stopping process('d Auzac *et al.*, 1989). The duration of latex flow, and thus in fact latex production, depends on the integrity of the lutoid when they flow out of the laticiferous tubes. Two type of parameters related to the state of lutoid or flow duration were reported ('d Auzac *et al.*, 1989). The first parameter is the bursting index (BI), determined by the ratio between the levels of lutoidic free acid phosphatase activity and total acid phosphatase activity after the complete brusting of lutoids by using a detergent (0.2% Triton-X 100). This ratio indicates approximate percentage of degraded lutoid. The BI is generally negatively correlated with the yield. The more the bursted lutoid, the lower the yield. The second parameter is plugging index (PI) which indicates the degree on rapid blocking capacity of the open extremity of the laticiferous tubes by formation of internal plugs and the thick external cap of coagulum. The higher the PI, the shorter the duration of flow and the lower the yield.

Following the observation that the enzyme acid phosphatase is released in the B serum when lutoids are damaged, Pujarniscle and Ribaillier (1966) considered that BI, which was inversely related to the osmolarity of the latex samples, indicated the percentage of the lutoids in a sample that had ruptured. Pujarniscle *et al.*, (1970) showed that the bursting

index of the first fraction of latex collected after tapping was higher than in subsequent fractions. This was in agreement with the finding of Pakianathan *et al.*, (1966) that damage to bottom fraction particles was greatest in the first flow fraction after tapping. Yeang and Paranjothy (1982), in studying seasonal variations in flow pattern, found high correlations between plugging index, (intensity of plugging) and bursting index.

Low (1978) noted a positive correlation between total cyclitols in C-serum and PI. He suggested that the higher osmotic pressure associated with higher cyclitol concentration could lead to a faster and greater dilution on tapping and hence to increase lutoid damage and fastering plugging.

There are three main effects which lead to lutoid breakage and changes in BI or PI as followed.

1.4.1 Mechanical effect.

The fragility of lutoid membranes accounting for the means of destabilization of these particles when they are subjected to substantial mechanical stress. This could happen during the fall in turgor pressure after the openning of latex vessels at tapping-cut. Accordingly, the highest level of shear effect is expected at the beginning of tapping when the fall in turgor pressure reaches its maximum. The early flow latex fraction was reported to be richer in damage lutoids (Pakinathan *et al.*, 1966). It was, however, suggested that at the same time the rapid swept flow of latex will exude the first microflocs out of the laticiferous system. It was probably only

in later phase, when flow became very slow, that damaged lutoids and rubber lutoid microflocs slowly began to plug the open extremities of latex vessels and cause the flow to stop (Yip and Southorn, 1968).

1.4.2 Osmotic effect

The dilution of fresh latex *in vitro* with increasing quantities of water, causes progressive damage to the latex bottom (lutoid) fraction. The damaged lutoid particles aggregate with rubber particles to form clusters which are lighter than lutoids (Pakianathan *et al.*, 1966). Osmotic changes in the vessel after tapping may in the general sense assist lutoid damage and lead to latex destabilization or coagulation which plug the open end of the latex vessels.

1.4.3 Electrical effect

The tapping wound gave rise to an action potential which might lead to depolarization of cellular or intracellular membranes. Such depolarization might act as a trigger for the release or leakage of solutes across membrane. It was reported that the formation of microflocs between rubber particles and damage lutoids are induced near the wounding site as a result of lutoid membrane leakage (Lim *et al.*, 1969).

1.5 The role of the lutoids in latex vessel plugging

Yip and Southorn (1968) produced evidence that lutoids could be disrupted by the mechanical shearing forces to which latex is subjected when flowing through the vessels under high pressure gradients

after tapping. Optical and electron micrographs showed distortion of particles along flow lines in the vessels, indicative of shear stress. When fresh latex was forced through glass capillaries of similar internal diameter to that of latex vessels, lutoids began to break down at pressure gradients above 0.2 atm/mm and at gradients of 0.4-1.2 atm/mm the capillaries plugged and flow stopped abruptly. With latex free from lutoids there was no plugging and flow could be maintained indefinetly even at 13 atm/mm pressure gradient. The evidence of these experiments is not conclusive since the conditions differ from those in latex vessels, but it is suggest that shear of lutoid could be a factor in plugging.

Studies of various workers have built up more evidences that a major cause of latex vessel plugging during latex flow is due to the damage of the lutoids. Microscopic examination of latex from the ultracentrifuge showed that dilution with water progressively damaged the bottom fraction particles (mainly lutoids) which could then aggregate with rubber particles to form flocs (Southorn, 1968). The lutoid damage was due to the osmotic shock. Similary, an evidence suggesting that changes in the osmotic concentration of the latex during flow after tapping could bring damage to lutoids (Yeang and Hashim, 1996). During the initial phase of fast flow, lutoids were swept out of vessels before they suffered irreversible damage. Although the permeability of lutoid membranes was partially affected, they could subsequently formed aggregates with rubber particles which were found in large numbers in the latex collected at the initial period

of the tapping cut. During subsequent, slower flow period, the lutoids suffered greater damage within the vessels themselves and aggregated with rubber particles to form flocs which accumulated near the cut ends, thus initiating the plugging process (Pakianathan *et al.*, 1966; Pakianathan and Milford, 1973).

Following the report on microscopic observation of plugs of rubber particles and damaged lutoids near the cut ends of vessels, Southorn and Edwin (1968) discovered that the fluid contents of the lutoids (B serum) caused rapid and complete flocculation of an aqueous suspension of rubber particles. In whole latex, breakage of lutoids by ultrasonic treatment resulted in the formation of flocs of rubber and damaged lutoids. They suggested that plugging within the vessels might be caused by the released B serum from ruptured lutoids (Yeang, 1989). Furthermore, fresh latex always contains micro-flocs, its formation was reported to be confined to the neighbourhood of damaged lutoids where the concentration of B serum in momentarily high, and to be limited by a stabilizing effect of C serum (Southorn and Edwin, 1968). Southorn and Yip (1968) envisaged fresh latex as a dual colloidal system with 2 major separated components; first, negatively charged particles (mainly rubber and lutoids) dispersed in the neutral C serum containning anionic proteins; and , second, a system within the lutoid membranes comprising the acidic B serum with metalic ions (especially Mg^{2+} and Ca^{2+}) and some cationic proteins. The two antagonistic systems can only exist so long as they are separated by the intact lutoid membranes. The release of the

B serum results in interaction between lutoid cationic contents and the anionic surfaces of the rubber particles, causing the formation of rubber flocs. The supportive evidence came from the fact that, intact lutoids underwent loss of polarization and increase in permeability prior to their disintegration.

1.6 Other factors involved in slowing down and stopping latex flow

It is possible that differences between clones in the composition of the protective film on the rubber particles may be partly responsible for differences in the case with which flocs are formed within the vessels. Rubber particles are surroundly protected by a complex film of protein and lipid material. Ho *et al.*, (1976) found marked differences between clones in neutral lipids concent of the rubber phase; on the other hand, phospholipid contents did not differ very much. These clonal differences are reflected in the values of acetone extract of total solids films (Subramaniam, 1976) and there appears to be a negative correlation with PI; long-flowing clones such as RRIM 501 have a high neutral lipid content in the rubber phase (as much as five times that of the high plugging clone Tjir 1). Sherief and Sethuraj (1978) found, in addition to a negative correlation between phospholipids of the lutoid membrane and bursting index, a negative correlation between triglyceride (the major neutral lipid) in the rubber phase and PI. Premakumari *et al.*, (1980) noticed that during wintering, when PI and BI increase, the neutral lipid content of the rubber particles decreased slightly,

as did the phospholipid content of the bottom fraction. These findings suggesting that, in addition to the effect of lutoid behaviour, plugging is influenced by the degree to which rubber particles are ' protected' by lipids.

While slowing down and cessation of flow after tapping seem to be mainly due to plugging within the vessels, it is likely that, at least toward the end of flow, coagulation on the cut also contributes. The formation of the external cap of coagulum (the 'tree lace') over the cut ends of the vessels may partly result from the aggregation of rubber particles and damaged lutoid. Moreover, a partial cause by the action of sap released from damaged bark cells was also suggested. This flocculating activity of the sap was reported by Cook and McMullen (1951). It has further been investigated by Gomez (1977) and by Yip and Gomez (1984). High molecular weight subtances in the sap were reported to be the major active components but the inorganic cation present (Na^+, K^+, Mg^{2+}, Ca^{2+}) were also essential for the flocculation of the latex particles. Gomez (1977) found that there were clonal differences in the flocculating activity of bark extracts.

1.7 Summary on current studies of latex vessels plugging

Despite several research advancements, the mechanism involved in latex flow stoppage after tapping is still not fully understood. It is clear that after the initial rapid drop in flow rate, the subsequent slowing up and cessation of flow is due to internal plugging of vessels near their cut ends and, perhaps, to the external cap of coagulum that forms over the cut ends.

The precise contributing mechanisim of these impediments has not been established, but it is likely that internal plugging is mainly responsible for slowing down flow, with the external cap playing a part towards the end of flow and eventually stopping it. The internal plugs consist of aggregates of rubber particles and damaged lutoids promoted to be formed by the flocculating effect of B serum released from ruptured lutiod. The lutoids are probably damaged both by osmotic effects and by the mechanical shearing forces to which the latex is facing when flowing through the vessels under high pressure gradients. During the initial stage of rapid flow, the latex is swept out of the vessels before there is irreversible damage to the lutoids; further damage occurs after the latex has left the vessels and some of the resulting flocs are retained on the cut. Later, when the flow rate is slower, flocculation occurs within the vessels and an increasing number of them become completely blocked.

2. Lectin

Lectins are ubiquitous natural proteins that hydrophobically bind carbohydrates with characteristic specificities. They have the ability to induce cell agglutination phenomena. Lectins are present in multiple molecular forms and have been mostly obtained in high yield and purity through conventional, affinity or high resolution chromatography. These proteins are of significant use in unravelling biological processes, clinical diagnostic systems and the elucidation of protein and carbohydrate structures (Kennedy *et al.*, 1995)

2.1 Definition

Lectins are naturally occurring protein and glycoproteins which selectively bind non-covalently to carbohydrate residues, The term "lectin" was first proposed by Boyd and Shapleigh in 1954. It is derived from the Latin verb "*legere*", which means to pick out, select or choose, and refers to the remarkable selectivity and specificity with which lectins recognize and bind to carbohydrate structures. It has largely superseded historical terms, such as " phytohemagglutinin" or "agglutinin" (originally used to describe the ability of lectins to cross-link and agglutinate cells).

The most widely accepted definition of the term "lectin" is that originally proposed by Goldstein *et al.*, (1980) and adopted by the Nomenclature Committee of the International Union of Biochemistry. This definition states that a lectin is "a carbohydrate binding protein of non-

immune origin, that agglutinates cells and/or precipitates polysacchrides or glycoconjugates"

This definition implies that lectin are :

- multivalent - two or more sugar binding sites are necessarry for the cross-linking of cells in agglutination, or of polysaccharides/ glycoconjugates in precipitation. However, in recent years, monovalent carbohydrate-binding molecules such as galectin 3 and adhesins have been described which are monovalent molecules but show multivalent behaviour when they are located close together at the cell surface;
- not antibodies - that they are of "non-immune origin" distinguishes lectins from antibodies against carbohydrate antigens, which can also act as agglutinins;
- not enzyme - the definition excludes most sugar binding enzymes (e.g. glycosidase, glycosyl transferases, glycosyl kinases) as they are monovalent, rather than polyvalent. In addition, given the right conditions, enzymes will catalyes a reaction so that sugars are modified – this will not happen when lectins bind to carbohydrate.
- distinct from certain toxins - for example, the toxic A chain of ricin, which possesses only sugar binding site, and act as an enzyme.

2.2 A brief history of lectinology

Stillmark was the first to describe the activity of lectin, when working on his doctoral thesis in 1887-1888 at the University of Dorpat in Estonia, one of the oldest university in Czarist Russia. His investigations were on the extract of seed from the *Euphorbiaceae* and a toxic protein, which he called ricin, from the seeds of the castor oil plant *Ricinus communis*. He studied their effects upon erythrocytes, liver cells, leukocytes and epithelial cells and noted agglutination reaction "like in clotting". Erythrocytes from different species reacted in different ways. Over next 40 years or so, a great many papers appeared describing dozens of new lectins (Kocourek, 1986). Owing to ease of availability, most were derived from plant sources, but lectin were also discovered subsequently to be present in bacteria, viruses, fungi, the blood and fluids of invertebrates, in the venom of snakes and even in man.

Stillmark, in his thesis of 1888, noted that the finding on effective serum in inhibition of hemagglutination by ricin. Other scientists followed up this observation, noting, for example, that gastric mucin had the same inhibitory effect (Landsteiner and Raubitschek, 1909). The significance of these observations were overlooked at the time, but they were the first indication of the carbohydrate-binding nature of lectins. Watkins and Morgan (1952) more than 40 years later were the first to show monosaccharides were capable of inhibiting lectin activity.

Landsteiner and Raubitschek (1907) were perhaps the first to note that lectins did not always agglutinate red blood cells of different individuals to an equal extent. It was not, however, until 30 years later that the reson for this became appearent with the first report of blood group-specific lectins. A pioneering paper was that of Boyd and Raguera (1949), which described blood group A-specific hemagglutination by an extract of Lima beans, *Phaseolus lunatus* syn. *limensis*. This led to enormous interest in the search for blood group-specific lectins and between 1945 and 1964 over 100 of them were discovered. The principle by which multivalent lectin binds to cell surface carbohydrate and cross-links cells in an agglutination reaction is illustrated in Fig. 5

Morgan and Watkins (1959) were the first to demonstrate that blood group specificity of lectin was as a direct result of their sugar binding specificity. They showed that hemagglutination by blood group A-specific lectin from *P.lunatus* syn. *limensis*, the Lima bean, could be inhibited by galNAc, while agglutination by blood group O-specific *Lotus tetragonolobus* lectin (LTA) could be inhibited by fucose. This was one of the first pieces of evidence for the presence of carbohydrates on the cell surface. The human A, B, and O blood group sugars are illustrated in Fig. 6

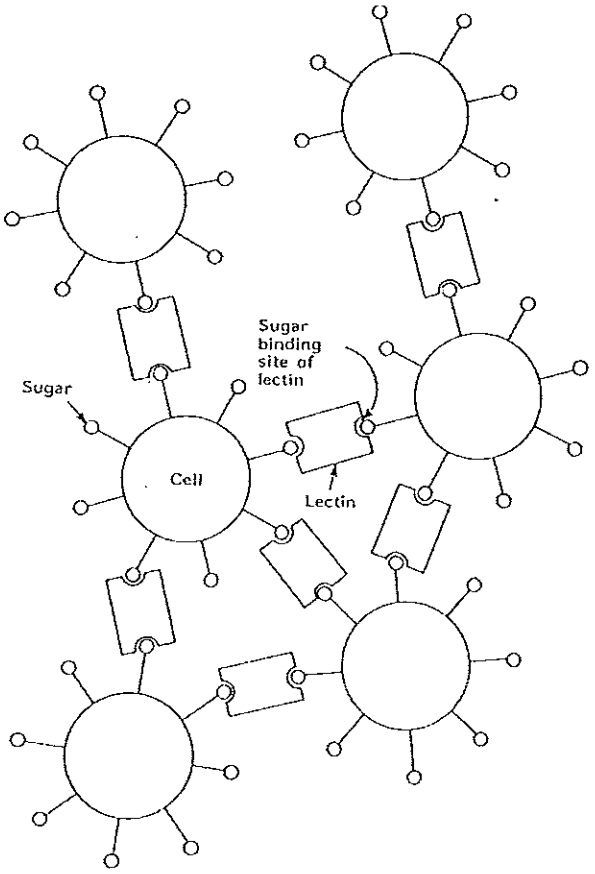


Fig. 5 Lectin-mediated cell agglutination. In this sample a divalent lectin binds to cell surface carbohydrates and cross-links the cells in an agglutination reaction. (Brooks *et al.*, 1997)

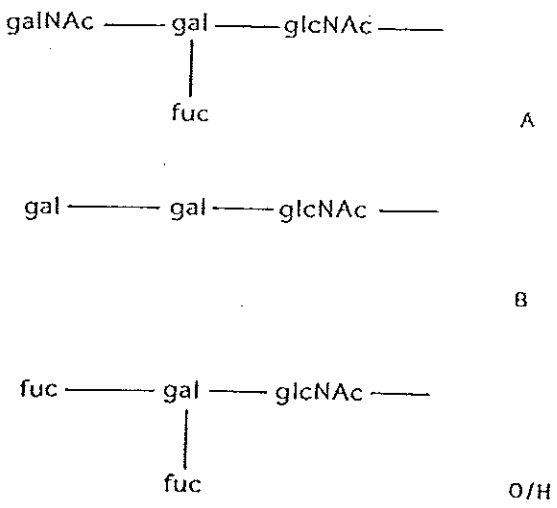


Fig. 6 Human A, B and O blood group sugars. (Brooks *et al.*, 1997)

2.3 Nomenclature of lectins

Lectins are usually named after their source ; either by its common name (e.g. peanut lectin) or by its Latin name (e.g. *Tetragonalobus purpureas* lectin). Often these names are abbreviated, for example soyabean lectin is often referrred to as SBA (which stands for soysbean agglutinin-agglutinin being a historical term for lectin); the lectin from *Helix pomatia*, the Roman snail, is commonly referred to as HPA (*Helix pomatis* agglutinin). Some abbreviations can be confusing, for example, the lectin from Phaseolus vulgaris is often, on historical groups, quite inappropriately known as PHA, which actually stands for phytohemagglutinin.

The literature also designates lectins in term of their mono-saccharide specificity e.g.D-galactose-binding hemagglutinin of *Pseudomonas aeruginosa* (Gilboa-Garber *et al.*, 1972); α-D-galactosyl-binding lectin from *Bandeiraea simplicifolia* (Hayes and Goldstein, 1974); D-mannose/D-glucose specific lectin from *Vicia cracca* (Baumann *et al.*, 1982) and D-galactose/N-acetyl-D-galactosamine-specific lectin from *Erythrina cristalli* (Iglesias *et al.*, 1982)

Other designations found for these proteins are endogenous lectins, used for vertebrate lectins present in specific cell types (Allen *et al.*, 1991); gal-lectins mentioned by Raz *et al.*, (1988), for endogenous lectins from normal and malignant tissues that bind D-galactosides; soluble lectins,

for proteins non-integrated with membranes (Jia and Wang, 1988), but with free movement in intra- and inter-cellular aqueous compartments (Barondes, 1984; Powell and Harrison, 1991).

2.4 Occurrence and sources of lectins

A general survey of the occurrence of lectins in different plant tissues indicates that the majority of these proteins are found in typical storage tissues. For example, most seed lectins are located in the parenchyma of the seed storage organs such as cotyledons (e.g. all legumes lectins; Van Driessche, 1988) and the endosperm (e.g. the lectins from *Ricinus commuis*; Lord, 1985). Similarly, most of the non-seed lectins occur in what can be called "vegetative storage" tissues as there are in tubers (e.g. potato; Allen, 1983), bulbs (e.g. tulip, Amaryllidaceae and Alliaceae species; Cammue *et al.*, 1986 and Van Damme *et al.*, 1991), corms (e.g. Araceae species; Sandhu *et al.*, 1990), rhizomes (e.g. *Aegopodium podagraria*, *Urtica dioica*; Peumans *et al.*, 1984a), root stocks (e.g. *Bryoniadioica*; Peumans *et al.*, 1984b) and bark (e.g. elderberry, black locust; Nsimba-Lubaki and Peumans, 1986; Nsimba-Lubaki *et al.*, 1986). Moreover, since leaves and stems of some plant species are also capable of accumulating storage proteins tissues. This is clearly the case for a plant like mistletoe (*Viscum album*), which accumulates large quantities of lectins in its leaves and (green) stems (Franz, 1989). The broad occurrence of lectins in different species, tissues or cells shows the importance of these molecules in nature.

2.5 Detection of lectins

The presence of lectins is mainly detected through a hemagglutination assay (Ohsawa *et al.*, 1990; Ozaeki *et al.*,1991). In this assay a serial dilution of the lectin is performed before an incubation with human or other animal erythrocytes. Additionally, to increase the sensitivity of the cells to lectin agglutination, an enzymatic (trypsin, papain or neuraminidase) or a chemical (glutaraldehyde or formaldehyde) pretreatment can be performed (Yanagi *et al.*, 1990) Other methods can also be used to identify lectin activity, such as a photometric assay (Teichberg *et al.*, 1988), precipitation of polysaccharides or glycoproteins (Delmotte and Goldstein, 1980; Shibuya *et al.*, 1989) and affinity electrophoresis (Goldstein and Hayes, 1978).

2.6 Binding specificity of lectins

Landstein and Raubischek (1908) were first to discuss the specificity of lectins and observed that several legume seed extracts promote different hemagglutination properties when assayed with erythrocytes from distinct animals. In relation to human erythrocytes, some lectins do not interact whereas others show weak agglutination properties or cell specificity. Lectins of the later type were isolated from Ulex europaeus-UEA 1, *Dolichos biflorus*, *Bandeiraea simplicifolia*-BSI-B and BSI-A4 as well as *Plecoglossus altivelis*. Specificity inside the Rh factor was a characteristic of *Clerodendro trichotomum* lectin (Bird and Wingham, 1968).

Lectins with specificity to different human blood cell types are more strongly inhibited by saccharides present in the immunodeterminant glycoproteins. For example, N-acetyl-D-galactosamine and L-fucose strongly inhibit lectins specific for type A and O erythrocytes, respectively. (Sharon and Lis 1972; Sikar and Chtterjee, 1990). Lectins non-specific to type A, B or O from the ABO system may exhibit mono and /or oligosaccharide specificity (Kellens *et al.*, 1989) In the case of a new lectin of undefined carbohydrate specificity, panels of cells and lectins of defined carbohydrate recognition can be used to unravel the nature of the lectin binding site (Cavalcanti *et al.*, 1990)

2.7 Molecular weight estimating of lectins

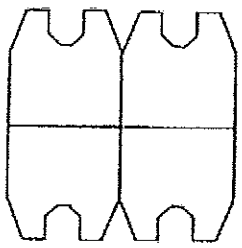
Molecular weight estimation of lectins is complicated due to readiness with which their sub-units undergo dissociation and association. These factors may in part account for the very large molecular weight range reported for plant lectins (40,000-270,000) (Sharon and Lis, 1972) . The majority, if not all lectins are composed of sub-units, which themselves may be composed of more than one polypeptide chain. In seed extracts of *Abrus precatorius* and *Ricinus communis*, there are both toxic, non-agglutinating, and non-toxic agglutinating lectins are found (Olsnes *et a*l., 1974a; Olsnes *et al.*, 1974b) The toxins, abrin and ricin both consist of two polypeptide chains of slightly different molecular weights joined by disulphide bridges. The A chains (molecular weights 30,000 and 32,000 in *Abrus* and *Ricinus* respectively) are termed "effectomers" since they carry the toxic activity,

manifested by their action on ribosomes and protein synthesis. The B chain (molecular weights 35,000 and 34,000 respectively), are termed "haptomers" since they actually bind to galactose-containing receptors on the cell surface through a single binding site. Abrin and ricin are therefore monovalent lectins, and any slight agglutinating activity found is due to their dimerisation, as appears to be the case for wheat germ agglutinating, which again only possesses one binding site per monomeric molecule (Levine *et al.,* 1972) . In contrast, the non-toxic *Abrus* and *Ricinus* agglutinins possess two binding sites per molecule. Native molecules have molecular weights of 134,000 (Abrus) and 120,000 (Ricinus) and is separated into four polypeptide chains by SDS and mercaptoethanol. The B polypeptide chains in the agglutinins, responsible for binding to cells are almost identical to those of the toxins, abrin and ricin. The possibility exists therefore that within one type of seed, the polypeptide chains involved in the binding of agglutinin and toxin to the cell surface receptors, are either identical, or very similar (Olsenes *et al.*, 1974).

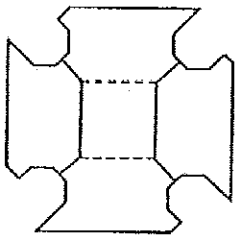
2.8 Structural organization of lectins and their interaction with carbohydrates.

There is a great diversity in lectin structural organization. The number of subunits per molecule is variable and the nature of polypeptide can be distinct (RCA I) or similar (wheat germ agglutinin, WGA), as shown in Fig. 7. Hydrophobic interactions, disulfide bridges and hydrogen bonds can be involved in the subunit association. The different polypeptides can have

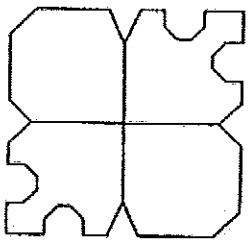
distinct functions. For example, RCA I contains two types of subunits, chain A and B; the enzymatic activity is in subunit A and the carbohydrate binding site is in subunit B (Houston and Doyle, 1982). Another characteristic of the broadness of lectin structure is the distribution of the carbohydrate binding sites per subunit. The nearby amino acid residues in the binding site was suggested to promote binding site formation (Quiocho, 1986). The ligand carbohydrate is first recognized by the carbohydrate binding sites of lectins and the binding interactions and followed through a lock and key type mechanisim, involving complex networks of the hydrogen bonds. The formation of a carbohydrate-protein complex involve the displacement of water molecules associated with the protein polar groups and around the highly polar sugar, with the establishment of new hydrogen bonds. These H-bondings and Van der Waals contacts are the dominant forces in binding stability (Quiocho, 1986).



Glycine max (Soy bean)



Triticum vulgaris (WGA)



Ricinus communis (RGA I)

Fig. 7 Samples on subunit structural organization in lectin (Kennedy *et al.*, 1995)

2.9 Lectins as cell recognition molecules

Recognition is a central event in a variety of biological phenomena and the first step in numerous processes based on cell-cell interactions, such as fertilization, embryogenesis, cell migration, organ formation, immune defense, and microbial infection. Improper functioning of cell recognition may cause disease. Thus, defects of leukocyte and platelet adhesion result in recurring bacterial infections and mucosal bleeding, respectively. Furthermore, aberrant cell recognition is thought to underlie the uncontrolled cell growth and motility that characterize neoplastic transformation and metastasis. An understanding of the molecular basis of the cell-surface code, therefore, has implications for intervention in many areas of biology and medicine (Sharon and Lis, 1989)

Cell recognition is thus another aspect of the fundamental concept of lock-and-key complementarity (Gilbert and Greenberg, 1984), which was originally formulated by Emil Fischer in 1897 to account for the specific interactions between enzymes and substrates. This hypothesis was extended by Paul Ehrlich (in 1900) and Frank Lillie (in 1914) to describe the interactions of cells with soluble molecules and with other cells, respectively. Thus, by the 1920s, the lock-and-key hypothesis had become one of the central theoretical assumptions of cellular biology. The nature of the

molecules involved in cellular recognition is still largely a mystery. During the past decades much attention has been focused on the possibility that such recognition is mediated by carbohydrates and lectins, a class of proteins of nonimmune origin that bind carbohydrates specifically and noncovalently (Liener *et al.*, 1986)

In the 1970s it became well established that almost all cells carry carbohydrates on their surfaces in the form of glycoproteins, glycolipids, and polysaccharides (Cook, 1986). Concurrently it was realized that carbohydrates have an enormous potential for encoding biological information (Sharon, 1975). In peptides and oligonucleotides, the information content is based only on the number of monomeric units and their sequence. Whereas in carbohydrates, information is also encoded in the position and anomeric configuration (α or β) of the glycosidic units and in the occurrence of branch points. Therefore, two molecules of a single monosaccharide (for example, glucose) can join to form 11 different disaccharides, but two molecules of a single amino acid or a single nucleotide can only form one dipeptide or one dinucleotide, respectively. Further structural diversification may occur by covalent attachment of sulfate, phosphate, and acetyl groups to the sugars. Thus, in theory, an enormous number of compounds can be derived from a relatively limited number of monosaccharides, leading to the hypothesis that

the specificity of many natural polymers is written in terms of sugar residues and not of amino acids or nucleotides (Sharon, 1975).

Although the existence of lectins has been known for more than 100 years (Sharon and Lis, 1987), the idea that they may act as recognition molecules is of recent development (Harrison and Chesterton, 1980). It was inspired by the realization that surface carbohydrates may function in cell recognition and was stimulated by advances in lectin research (Liner *et al.*, 1986). In particular, it was demonstrated that these proteins are not confined to plants, as originally believed, but are ubiquitous in nature, being frequently found on cell surfaces and intracellular particles. Several membrane-bound lectins were shown to participate in the selective uptake of glycoproteins into cells in the intracellular trafficking of glycoproteins (Ashwell and Morell, 1982). In addition, characteristic changes in lectin expression that coincide with distinct physiological change in the living cells or tissues were observed. The specificity of lectins proved to be very exquisite than originally assumed, since they can not only distinguish between different monosaccharides, but also specificically bind to oligosaccharides, detecting subtle difference in complex carbohydrate structures. Finally, speed and reversibility of lectin-carbohydrate interactions satisfy extra requirements expected for a cellular recognition system.

Typically, the lectin and the complementary carbohydrate are located on the surfaces of apposing cells, which may be of the same type or of

different types. Cell may also interact via bridges formed by soluble glycoprotein that bind to the cell surface lectins. Alternatively, the lectins may combine with carbohydrates of insoluble components of the extracellular matrix that promote cell substrate adhesion. In addition, soluble lectins may act as bridges by binding to carbohydrates on apposing cells.

Various experimental approaches have been reported to demonstrate the participation of lectins in cell recognition (Ofek *et al.*, 1985). Many are indirect and based on the assumption that whenever such recognition is carbohydrate-dependent, a lectin must be involved. The carbohydrate dependence of a cell-cell interaction can be demonstrated by methods such as inhibition by sugars or glycoconjugates, enhancement or inhibition by enzymatic or chemical modification of cell surface carbohydrates, or the use of mutant cells, for example, lectin-resistant mutants with altered composition or structure of surface carbohydrates. However, such evidence does not by itself exclude the participation of other types of carbohydrate-binding protein, for instance, cell surface glycosyltransferases, in cell recognition (Hathaway and Schur, 1988). To obtain direct evidence, it is essential to isolate the putative lectin and its ligand and establish that they indeed take part in the recognition phenomennon. This should include a demonstration of their surface location and of the ability of the purified lectin and ligand, as well as of antibodies to the lectin and to the ligand, to specifically block the interaction between cells. In addition, changes in the

amount of the lectin should be closely linked to physiological events for which cell-cell recognition is required. Besides carbohydrate-binding sites, lectins may contain sites specific for noncarbohydrate ligand, and these sites, too, may be critical for the recognition functions of the lectin (Barondes, 1988).

Plant lectins were among the first proteins of this class to be studied due to their broad distribution and ease of isolation. More lectins have been characterized from plants than from any other sources (Goldstein and Poretz, 1986). However, little is known concerning their function. The only hypothesis currently attracting attention is that they serve as mediators of the symbiosis between nitrogen-fixing microorganisms, primarily rhizobia, and leguminous plants, a process of immense importance in both the nitrogen cycle of terrestrial life and in agriculture.

The association between legumes and nitrogen-fixing bacteria is highly specific. For example, rhizobia that infect and nodulate soybeans cannot nodulate garden peas or white clover, and vice versa. The idea that lectins are responsible for this association was initially based on the finding that a lectin from a particular legume, for example, soybean agglutinin bind in a sugar-specific manner to the corresponding rhizobial specices and not to bacteria that are symbionts of other legumes (Hamblin and Kent, 1974). A similar specificity pattern was observed with lectins from soybean, pea, red kidney bean, and jack bean seeds and lipopolysaccharides from the respective symbionts (Etzler, 1985). Although the lectins used were isolated from seeds

and there was no proof at the time that roots contain lectins with similar specificities. It was suggested that rhizobial attachment to plant roots occurs by direct interaction between bacterial surface carbohydrate and lectins present in the root.

There is convincing evidence in favor of the lectin recognition hypothesis in the symbiosis between white clover, *Trifolium repens*, and *R. trifolii* (Dazzo and Truchet, 1984). A lectin (trifoliin A) specific for 2-deoxyglucose was isolated from extract of clover seeds and seedling roots. It bound to infective, but not to uninfective, strains of *Rhizobia*. Antibodies to the lectin bound mostly to the root hair region of clover roots but did not bind to roots of other closely related legumes, for example alfalfa. The lectin was released from the roots by the sugar for which it is specific, suggesting that it associates with the root surface via its carbohydrate-binding site. Trifoliin may thus act as a bridge between similar carbohydrate on both the root hair tips and *R. trifolii*. A polysaccharide that could serve as such a receptor is present on the surface of infective stains of *R. trifolii* but absent (or inaccessible) on noninfective strains.

Other findings, however, cast doubt on the general validity of the notion that lectins serve as recognition molecules in host-symbiont interactions in leguminous plants (Etzler, 1985). For instance, *R. leguminosarum*, a symbiont of peas, binds to the root hair tips not only of pea but also of other leguminous plants, such as *Canavalia ensiformis* and

Medicago sativa. The latter, however, are not infected by this bacterium. In addition, heterologous rhizobia attached to pea root hair tips nearly as well as did *R. leguminosarum*. Finally, sugars specific for pea lectin do not inhibit the attachment of *R. leguminosarum* to root hairs. Thus the lectin recognition hypothesis continues to be the subject of controversy.

2.10 Classification lectin

The lectin can be classified into two groups-soluble lectins that may play a role in the secretion or organization of extracellular glycoconjugates (Barondes, 1984) and membrane lectin that are intergrated into membranes and are appearently involved in translocation of glyco-conjugates in cells (Ashwell and Harford, 1982). The soluble lectins are extracted from tissues with simple buffers, often fortified with complementary sugars that may dissociate them from membrane-bound glycoprotein conjugates. The membrane lectins require detergents for solubilization, although they may then remain soluble in their absence.

The group of membrane lectins is belived to be integrated in membranes, although, in most cases, this has not been rigorously established. The starting material for their preparation may be either an acetone powder of the tissue or a crude membrane fraction and nonionic detergents such as Triton X-100 are generally used for solubilization. The membrane lectins are generally assayed by formation of precipitable complexes with soluble glycoconjugates rather than as hemagglutinations. However, the first such

protein to be characterized extensively was shown to agglutinate both untreated and sialidase-treated erythrocytes (Stockert *et al.*, 1974) and, like many classical lectins, also induced mitosis in lymphocytes (Novogrodsky and Ashwell, 1977). Most of the known membrane lectins require Ca^{2+} for their carbohydrate-binding activity, whereas most of the soluble lectins do not.

The first of this group of carbohydrate-binding proteins was discovered in the course of studies of the uptake of circulated glycoproteins by rabbit liver. It had been observed (Morell *et al.*, 1968) that removal of terminal sialic acid groups from the serum glycoprotein ceruloplasmin led to rapid uptake of this protein by the liver. In a series of studies, summarized by Aswell and Morell (1974), it was shown that the galactose residue exposed by desialylation is critical for hepatic uptake, suggesting the presence of a galactose-binding protein on the surface of liver cells. Such a protein was subsequently found in Triton X-100 extracts of rabbit liver, and this first membrane lectin was then purified by affinity chromatography on asialo-orosomucoid linked to sepharose (Hudgin *et al.*, 1974).

The membrane lectins generally believed to participate in shuttling soluble glycoproteins from the outside to the interior of the cell and within intracellular compartments. In the case of the phosphomanosyl receptor the current view is that it directs glycoproteins containing manose-6-phosphate residues within intracellular compartments to lysosomes. The

asialo- glycoprotein receptor concentrated on the hepatocyte cell surface is generally believed to be involved this uptake. In some cases uptake leads to degradation but in others it does not. As with the soluble lectins, it is quite possible that the same protein may serve more than one function, and that the specific function may be dictated not only by the intrinsic properties of the carbohydrate-binding protein but also by those of the complememtary glycoconjugates to which it is exposed (Barondes, 1986).

2.11 Isolation and purification of lectins

There are no general strategies for the isolation of lectins. The isolation procedure(s) are usually dictated by the source (seed, serum, bacteria or other) and may involve classic protein purification scheme. Some extracts may be initially fractionated by ammonium sulfate and then by ion-exchange chromatography. If the specificity of a lectin is known, the lectin may be purified to homogeneity on affinity sorbents. Elution of lectin can then be realized by use of solutions of saccharide or chaotropes, or by lowering the pH. It is essential that eluting agents be removed from the purified lectin(s) and that the agents do not irreversibly alter the saccharide-binding site(s). Affinity methods rarely separate isolectins, although a combination of affinity and ion-exchange techniques may afford reasonable separation.

For studies on memebrane, the single most important feature to ascertain about a membrane protein at the outset is its mode of association with the bilayer (Fig. 8) This information will largely govern the methods to be adopted for its purification. If integrated into the hydrophobic phase, liberation of the so-called *integral* (or intrinsic) membrane proteins in soluble form will require disruption of the phospholipid bilayer or cleavage of the polypeptide from its membrane anchor. During all the subsequent purification procedures, 'shielding' of any subtantail intramembranous hydrophobic face from the aqueous media will be required; this is usually achieved with detergents. *Peripheral* (or extrinsic) membrane proteins, on the other hand, are associated with the membrane surface through interactions either with other proteins or with the exposed regions of phospholipid. In these case, the protein may often be dissociated from the membrane by altering the ionic conditions of the buffer or , in more resilient situations, by inducing a degree of denaturation. Although detergents can help liberate this type of protein from the membrane, they are rarely required subsequently.

Separation of membrane fractions or subcellular organelles can be achieved by a number of different approaches. The most successful of these is centrifugation , normally using a density gradient to some kind. Other approaches which have been used to advantage include two polymer phase partitioning, high-voltage free-flow electrophoresis and (immuno) affinity and gel-filtration chromatography. Considerable patient application and ingenuity

has gone into membrane isolation, and good methods now exist for most organelles and membrane systems.

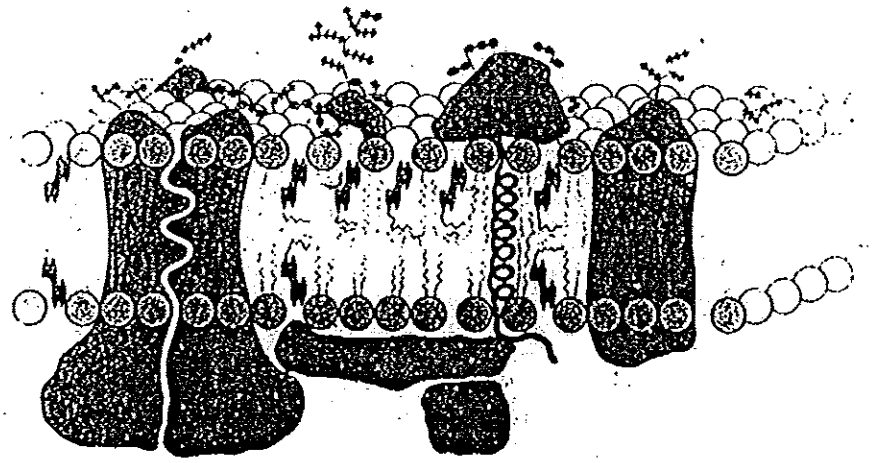


Fig. 8 Schematic diagram of generalized membrane structure. The membrane protein can be categorized into two classes : integral and peripheral. The latter group are considered not to make hydrophobic contacts with the bilayer. Both group of proteins can exist as monomers or as hetero- or homo-oligomers. Carbohydrate residues (♦) are shown on the extracelluar surface of the membrane. (Findlay, 1990)

Most membrane-bound proteins can be extracted from their membrane by detergents possessing lipopholic chains which bind to the protein at its hydrophobic surface in lieu of the normal membrane. Of the detergents that can be used for this propose, sodium deoxycholate and Triton are widely used. Triton is the trade name for a series of polyethyleneglycol-based, mostly nonionic detergents, of which the most widely used for a variety of purposes is Triton X-100 (Fig. 9), although other types have specific uses.

Detergents are capable of displacing a protein which is tightly bound by hydrophobic forces in membrane, firstly by dissovling the membrane, and secondly by replacing the membrane by aliphatic or aromatic chains which form the lipophilic part of the detergent (Fig. 10).

Once the protein has been solubilized and the enzyme activity must be maintained ; this can be a matter of choosing a suitable detergent. Non-ionic detergents such as Triton X-100 are mild in their action, and most proteins, whether originally membrane-bound or not, can tolerate Triton at levels of 1-3% w/v. at the other extreme, some anionic detergents (e.g. dodecyl sulfate) are extremely denaturing, so although efficient solubilizer, will probably not be much use in protein enzyme isolation.

Excess detergent can interfere with fractionation. For instance, salting out with ammonium sulfate causes Triton X-100 to separate as a floating layer, frequently containing the proteins required, without resulting in

any useful separation. Column chromatography can be carried out; gel filtration may separate the proteins, but detergent micells can travel in the same area remain with the protein. Ion exchange chromatography is quite successful in the presence of nonionic detergents. Indeed, with normal water-soluble proteins the presence of up to 1% Triton X-100 seem to make little difference to ion exchange behavior. But membrane-solubilized proteins may be present only in detergent micelles which influence ion exchange behavior substantially. If the protein required can be persuaded to adsorb to an ion exchanger, excess detergent will pass through; then elution of the (relatively) detergent-free protein can occur. On the other hand, if loss of all detergent results in denaturation, a small concentration (<0.1%) can be included in the buffer to prevent this; the eluted fraction will of course again contain some detergent.

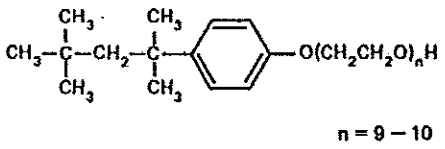


Fig. 9. Structure of Triton X-100

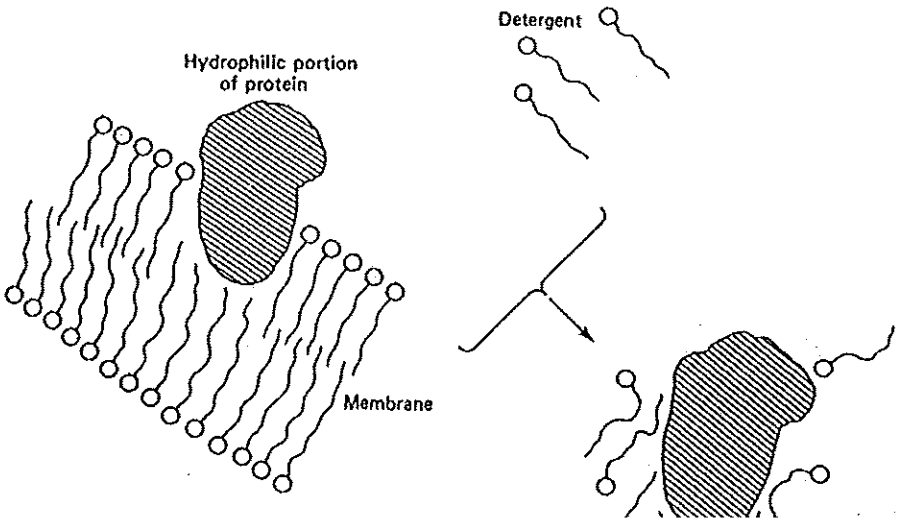


Fig. 10 Action of detergent in solubilizing membrane-located proteins. The solubilized protein may be as illustrted or bound in a more complex detergent-micelle, depending on the relative proportions of proteins and detergents (Scopes, 1994)

Objective

The objectives of this study are :

1. To isolate and purify *Hevea* latex lectin and *Hevea* latex lectin binding protein from latex of *Hevea brasiliensis*.
2. To study biochemical characteristics of *Hevea* latex lectin and *Hevea* latex lectin binding protein.
3. To study binding specificity between *Hevea* latex lectin and *Hevea* latex lectin binding protein.
4. To study interaction between *Hevea* latex lectin and rubber particles in inducing rubber particles aggregation.
5. To study the effect of *Hevea* latex lectin binding protein in rubber particles aggregation induced by *Hevea* latex lectin.
6. To study the correlation between activity levels *Hevea* latex lectin binding protein and fresh rubber latex yield per tapping.

Chapter 2

Materials and Methods

Materials

1. Chemicals

Acrylamide, ammonium persulfate, ammonium sulfate, acetone, asialofetuin, asialomucin, α-1-acid glycoprotein, bovine serum albumin, Bio-Gel P-300, Biolyte 3/10, catalase, carbonic anhydrase, chicken egg albumin, chitin, coomassie blue R, DEAE-Sepharose, DEAE-Sephacel, dextrose, fetuin, ferritin, Folin phenol reagent, glycine, D-glucose, glucosamine, lactate dehydrogenase, lactose, α-lactalbumin, 2-mercaptoethanol, melibiose, N-acetylgalactosamine, N-acetylglucosamine, N-acetylmanosamine, N-acetylneuraminic acid, N,N'-methylene bis-acrylamide, N,N,N',N'-tetramethylenediamine (TEMED), mucin, ovalbumin, phophorylase b, phenol, Sepharose 6B, sodium chloride, sodium dodecyl sulfate, sodium hydroxide, sucrose, SM2, Tris (hydroxymethylaminomethane), Tirton X-100, trypsin inhibitor.

2. Instruments

2.1 CE 272 lilnear ultraviolet spectrophotometer series 2

2.2 Ultracentrifuge model L5-65 (Beckman)

2.3 Centrifuge model TJ-6 (Beckman)

2.4 Deep-freeze refrigerator (Scientemp)

2.5 Fraction collector model 2110 (Bio-Rad)

2.6 Microcentrifuge model H-3 (Kokusan)

2.7 Micro tube pump model MP-3

2.8 Power supply model 3200

2.9 Speedvac concentrator SVC 100 (Savant)

2.10 Micro hematocrit centrifuge (ICE MB centrifuge)

Methods

1. Collection of latex from rubber tree

The latex used in this study was obtained from rubber trees of *Hevea brasiliensis* (Clone RRIM 600) under the age of 20 years, grown at Songkhla Rubber Research Institute, Hat Yai, Songkhla. The trees were tapped in a half-spiral fashion every other day at 6.00 a.m. Rubber tapping was performed with V-shape (V-bong) knife by strpping *Hevea* bark to make cut across latex vessels, located within the bark area 3-8 mm away from the outer cork layer of trees. The exuded latex was collected in plastic containers which

were chilled on ice. Latex collection period of forty-five minutes was done on each tapping tree.

2. Separation of latex by ultracentrifugation

The chilled latex was filtered through 4 layers of cheese cloth to remove the particulate materials and bark tissue debris. The filtrate was collected and centrifuged at 59,000 g for 45 min. at 4 ^{o}C in an ultracentrifuge (Beckman model L8-70M). After being centrifuged, the latex was separated into 4 distinct layers depend on different density of compounds containing in the latex. The top layer was a white creamy layer of rubber, the next layer underneath was yellowish and called Frey-Wyssling, the middle layer was a clear solution called C-serum and the pellet was bottom fraction which mainly comprised with lutoid particles as shown in Fig. 11.

3. Extraction of *Hevea* latex lectin from bottom membrane

3.1 Method A

The bottom (lutoid) fraction (35 g), separated from ultra-centrifuged fresh latex of 350 ml, was washed by suspending in a 5 volumes of Tris-buffed saline, TBS (50 mM Tris-HCl pH 7.4 containing 0.9% NaCl) with gently stirring for 30 min at 4 ^{o}C, was then separated

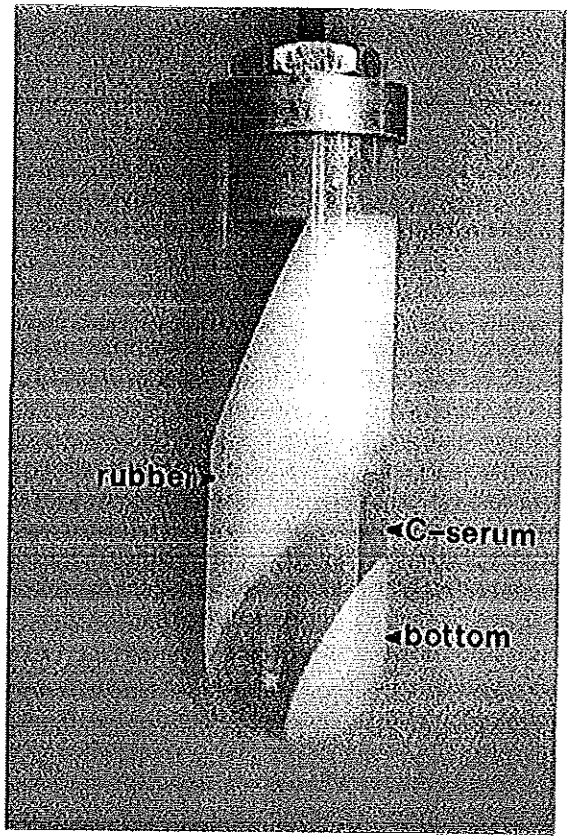


Fig. 11 Ultracentrifuged fresh latex.

from contaminated C-serum and rubber particless by ultracentrifugation 30,000 g for 30 min. The washing was repeated 3 times. The washed lutoid fraction was then bursted by addition of cold acetone to reach 30% saturation. The mixture was stirred and kept on ice for 30 min. The membrane pellet fraction was isolated by centrifugation 3,000 g for 10 min and washed with TBS to eliminate residual B-serum. The washed membrane pellet fraction was extracted by suspending in 3 volume of TBS containing 0.2% Triton X-100. The mixture was stirred at 4 ^{o}C overnight. The *Hevea* latex lectin (HLL) extract was recovered after centrifugation at 10,000 g for 25 min and concentrated by centrifugation under 30,000 NMWL membrane cut off.

3.2 Method B

The bottom (lutoid) fraction (100 g), separated from ultra-centrifuged fresh latex of 1,000 ml, was washed by suspending in a 5 volumes of TBS with gentle stirring for 30 min at 4 ^{o}C. It was then recovered from contaminated C-serum and rubber particles by ultra-centrifugation 30,000 g for 30 min. The washing was repeated 3 times. The washed lutoid was bursted by mixing with an equal volume of distilled water and stirred overnight at 4 ^{o}C. The lutoid membrane was collected by centrifugation 10,000 g for 30 min and then washed with TBS to eliminate residual B-serum. The lutoid membrane fraction was extracted by suspending

in 10 volumes of 50 mM Tris-HCl pH 7.4 containing 0.5% Triton X-100. The mixture was stirred at 4 °C overnight and the HLL extract recovered after centrifugation at 10,000 g for 30 min. Two-phase separation was performed on the extract by adding ammonium sulfate to reach 40% saturation and stirred 1 hour. The upper phase was collected by centrifugation 5,000 g for 10 min and fractionated by addition of 3 volumes of cold acetone. The pellet was recovered after centrifugation 5,000 g for 5 min and extracted in TBS containing 0.2% Triton X-100 by stirring overnight. The HLL extract was obtained after centrifugation at 30,000 g for 30 min.

4. Purification of *Hevea* latex lectin from bottom fraction

4.1 Chitin batch-binding

The concentrated HLL extract (8 ml) from Method A was incubated with M2 (1:10,w/v) for 15 min to remove residual Triton X-100. It was mixed with 10 ml of chitin solution containing 3 g of solid weight. The mixture was shaked at 4 °C overnight. The unbound chitin fraction was removed by centrifugation 10,000 g for 10 min. The bound fraction was washed in 3 volumes of 50 mM Tris-HCl pH 7.4 (buffer A) for 3 times and followed by stepwised elutions with buffer A containing 0.5 M NaCl and 0.2% Triton X-100, respectively. The fractions were screened for

hemagglutination activity. The active HLL fractions were pooled and used for further purification steps.

4.2 DEAE-Sepharose CL-6B chromatography

Either the HLL fraction eluted from chitin under 4.1 or the HLL extraction from Method B (with previous incubation with SM2 (1:10,w/v) for 15 min to remove residual Triton X-100) was applied onto a column (1.5x5 cm and 1.5x 12 cm) of DEAE-Sepharose CL-6B, previously equilibrated with 50 mM Tris-HCl pH 7.4 (buffer A) at a flow rate of 15 ml/h. The column was washed with buffer A until the absorbance at 280 nm was below 0.01. Stepwised elutions were then followed by using buffer A containing 0.5 M NaCl and 0.2% Triton X-100, respectively. Each fraction was screened for hemagglutination activity and the active HLL fractions were pooled and concentrated for further characterization studies.

5. Preparation of C-serum *Hevea* latex lectin binding protein

The C-serum (60 ml) obtained from ultracentrifuged fresh latex was fractionated with ammonium sulfate. The protein pellet obtained between 70-85% of salt saturation was recovered by centrifugation 15,000 g for 20 min and dissolved in buffer A. Two volumes of cold acetone was added into the protein solution, mixed well and kept on ice for 5 min. The resulting pellet was collected by centrifugation 10,000 g for 5 min at 4° C

and dissolved in the same buffer. The soluble supernatant fraction containing C-serum *Hevea* latex lectin binding protein (CS-HLLBP) was separated by centrifugation at 30,000 g for 10 min at 4°C. The H.I. activity of CS-HLLBP was measured, concentrated and further purified on column chromatography.

The activity of CS-HLLBP was measured from its ability to inhibit hemagglutination induced by HLL. Each 25 µl of CS-HLLBP sample was serially diluted with 25 µl of TBS in a microtiter U plate. This is followed by the addition of 25 µl of HLL sample possessed hemagglutination units (titer) of 4, the solution was mixed and incubated at room temperature for 20 min before the addition of 25 µl of 2% rabbit erythrocyte suspension into each well. Hemagglutination inhibition activity was recorded, after incubation for 1 hour at room temperature, as the minimum concentration of inhibitors which completely inhibit HLL activity. The inhibition activity was expressed in term of hemagglutination inhibition (H.I.) unit or titer.

6. Purification of C-serum *Hevea* latex lectin binding protein

6.1 Bio-Gel P 300 chromatography

The concentrated crude CS-HLLBP (3 ml) solution, obtained as described under No. 5, was loaded onto a Bio-Gel P 300 column (1.8x80 cm), previously equilibrated with buffer A the column was eluted with the same buffer at a flow rate of 12 ml/h. Fractions (3 ml) were collected and assayed for H.I. activity. The active fractions were pooled, concentrated and further purified on ion-exchange column chromatography.

6.2 DEAE-Sephacel column chromatography

The CS-HLLBP active fraction pooled from Bio-Gel P 300 was applied onto a column (1.8x 8 ml) of DEAE-Sephacel previously equilibrated with buffer A. The column was washed at a flow rate of 15ml/h. with buffer A until the absorbance at 280 nm was below 0.01 and stepwise-eluted with buffer A containing 0.1 and 0.2 M NaCl, respectively. The fractions (3 ml) were collected and assayed for H.I. activity. The active fractions were pooled, desalted, concentrated and used for further characterization studies.

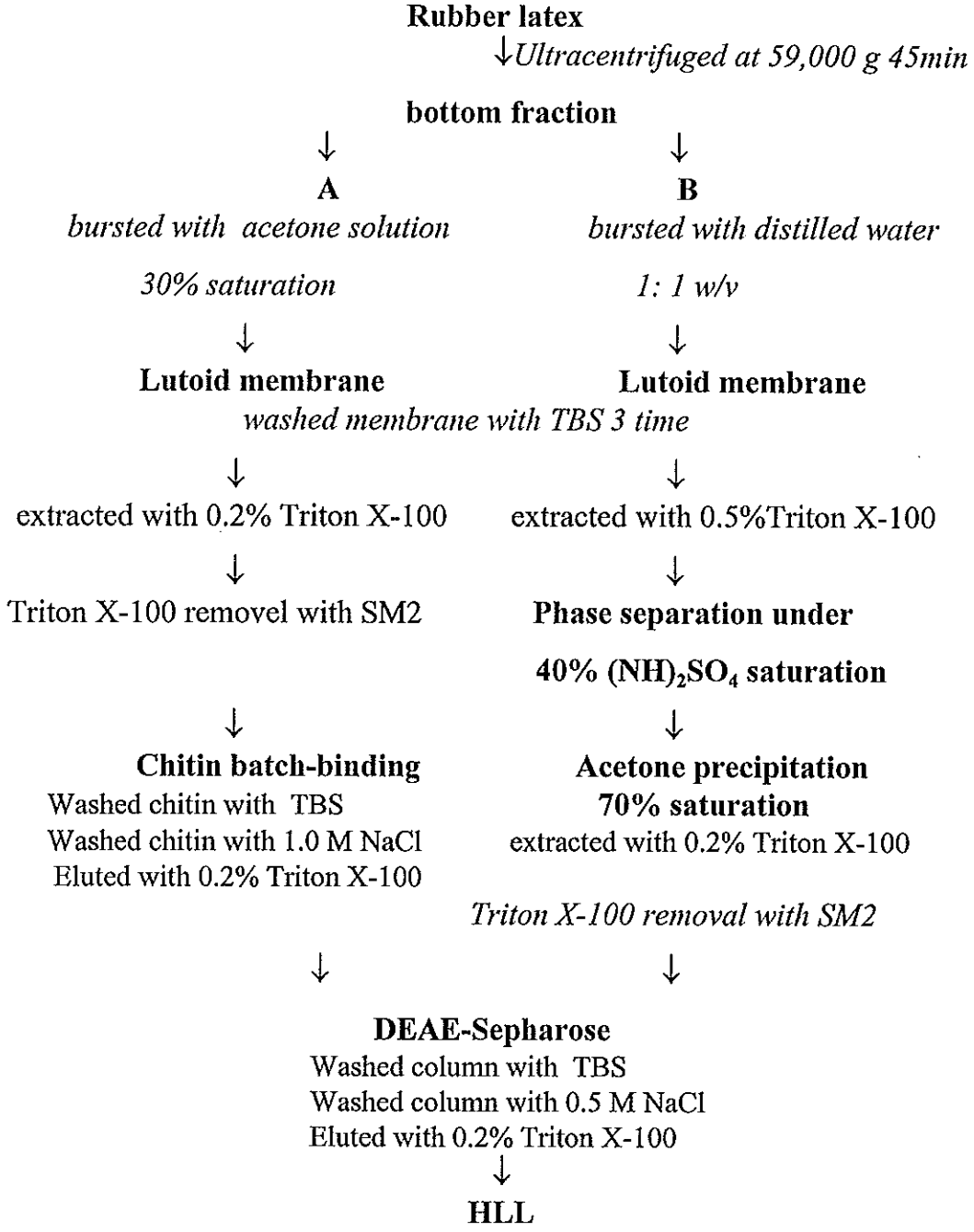


Fig. 12 Summarized diagram for purification of HLL under Method A and B.

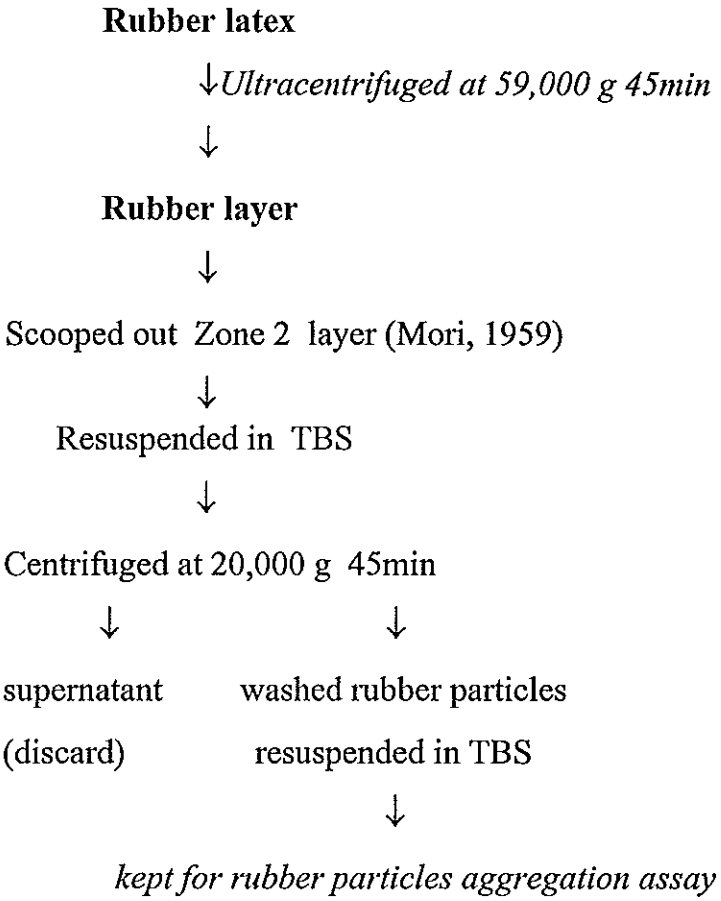


Fig. 13 Summarized diagram for preparation rubber particles.

Rubber latex

↓

Ultracentrifuged at 59,000 g 45min

↓

C-serum

↓

$(NH)_2SO_4$ precipitation (70-85 %)

↓

pellet

(dissolved in 50 mM Tris-HCl pH 7.4)

↓

Acetone precipitation (70% saturation)

↓

pellet

(dissolved in 50 mM Tirs-HCl pH 7.4)

↓

Bio-Gel P-300

↓

DEAE-Sephacel

↓ eluted @ 0.2 M NaCl

CS-HLLBP

Fig. 14 Summarized diagram for purification of CS-HLLBP

7. Preparation of erythrocyte suspension

Rabbit blood (5 ml) was collected by veinpuncture and mixed with 1 ml of acid citrate dextose anticoagulant (0.075 mM trisodium citrate, 0.038 M citric acid and 0.12 M dextrose) and stored at 4 ^{o}C up to 3 days. Erythrocyte suspension was freshly prepared by washing the erythrocytes three times with 10 volumes of TBS and then suspended in the same buffer to make a 2% (V/V) suspension.

8. Hemagglutination assay

The hemagglutination activity of lectin was performed by using a microtiter plate U shape (microwell plate 96 U;Nunc). A lectin sample (50 µl) was serially diluted with 50 µl of Tris-buffered saline, TBS (50 mM Tris-HCl pH 7.4 containing 0.9% NaCl) and mixed with 50 µl of a 2% rabbit erythocyte suspension. This reaction plate was incubated at room temperature for 1 hour before recording the hemagglutination activity. The HLL activity was expressed in term of hemagglutination unit (H.A. unit or titer) which was the reciprocal of the lowest dilution of HLL giving complete hemagglutination. A control was carried out in the same manner but buffer was used in stead of the HLL. The 4 H.A. unit of HLL was prepared from suitable HLL concentration and used for hemagglutination inhibition assay as described under the following Method.

9. Hemagglutination inhibition assay

C-serum *Hevea* latex lectin binding protein (CS-HLLBP), monosaccharides, oligosaccharides, saccharide derivatives and glycoproteins (fetuin, asialofetuin, mucin, asialomucin, ovomucoid and trypsin inhibitor type II-S) were tested for inhibition of hemagglutination by HLL. Each inhibitor sample (25 µl) was serially diluted with 25 µl of TBS. An aliquot 25 µl of HLL sample containing 4 H.A. units was then added. The mixture was incubated at room temperature for 20 min before the addition of 50 µl of 2% (v/v) rabbit erythrocyte suspension to each wells. Hemagglutination was recorded after further incubation for 1 h at room temperature. The inhibition activity was expressed in term of the lowest dilution of inhibition unit (H.I.) titer or unit defined as the reciprocal of the lowest dilution of inhibitors which completely inhibited hemagglutination activity of HLL. The control assay was performed by replacing inhibitor sample with TBS.

10. Procedure for subunit molecular weight determination by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

Polyacrylamide gel electrophoresis was carried out according to Laemmli (1972) in the presence of 0.1% SDS using a slab gel (10x10x0.1cm). The separating slab gel (10x8x0.1 cm) containing 12% of

acrylamide concentration and the stacking gel (10x2x0.1 cm) containing 3 % of acrylamide. The composition of both separation and stacking gels were summarized in Table 1. The standard molecular weight markers (Pharmacia, Sweden) used were phosphorylase b (94 kD), albumin (67 kD), ovalbumin (43 kD), carbonic anhydrase (30 kD), trypsin inhibitor (20 kD) and α-lactalbumin (14.4 kD).

Table 1 The composition of separating and stacking gels

Reagents (µl)	Stacking gel 3%	separating gel	
		10%	12%
30% acrylamide + 0.8% bis-acrylamide	300	2000	2400
1.5 M Tris- HCl pH 8.9	-	1500	1500
0.5 M Tris- HCl pH 6.8	750	-	-
1% SDS	300	600	600
Distilled water	1570	1740	1340
1% Ammonium persulfate	75	75	75
TEMED	5	5	5
Total volume	3000	6000	6000

10.1 Preparation of sample for SDS-PAGE

The samples were treated with solubilizing buffer with final concentration of 0.0625 M Tris-HCl buffer pH 6.8, 2% SDS, 10 % glycerol, 5% mercaptoethanol and 0.001% bromophenol blue as the dye. Before applying the sample, the proteins were dissociated by immersing the sample for 5 min in boiling water.

10.2 Running condition

The SDS-PAGE was carried out at room temperature with the anode in the lower chamber, and the electrode buffer pH 8.3 was comprised of 0.025 M Tris-HCl, 0.193 M glycine and 0.1% SDS. An electrical current of 14 mA was applied until the tracking dye marker approached the bottom of gel.

After the electrophoresis was completed, the slab gel was fixed and stained with Coomassie brilliant blue R250 staining solution for detection of protein bands. The molecular weight of each protein sample was determined from a plot of relative migration values (R_f) against the log MW of standard markers. R_f was defined as:

$$R_f = \frac{\text{Distance protein has migrated from origin}}{\text{Distance form origin to reference point (the tracking dye)}}$$

10.3 Coomassie staining

The slab gel was fixed and stained for 2 hours with 0.2% Coomassie brilliant blue R in 50% methanol and 7% acetic acid at room temperature. Destaining of excess dye was removed by repetitive changing of destaining solution containing 20% methanol and 10% acetic acid until the clear background was obtained. The molecular weight of each protein sample was determined from the plot of migration distance against the log MW of standard markers.

11. Native molecular weight estimation of *Hevea* latex lectin

The molecular weight of native HLL was determined by gel filtration on a column of Sepharose 6 B (2.5 x 85 cm) in presence of 0.1 % Triton X-100. The 2 ml fractions were collected at the flow rate of 15 ml/h. A pooled the active fractions from DEAE-Sepharose column under No. 4.2 was used as native HLL sample. The standard markers used for MW. determination were blue dextran (2,000 KD), aldolase (158 kD), catalase (240 kD), ferritin (440 kD), thyroglobulin (660 kD) and potassium dichromate (294 D). A plot of elution volume versus the log of the MW of these standard markers will give a standard curve for calibrating the unknown MW of the native protein.

12. Native for molecular weight estimation of C-serum *Hevea* latex lectin binding protein

The molecular weight of native CS-HLLBP was determined by gel filtration on column of Sepharose 6 B (2.5 x 85 cm). The 2 ml fractions were collected at the flow rate of 15ml/h. An eluted CS-HLLBP active fraction from DEAE-Sephacel column under No. 6.2 was used as native CS-HLLBP sample. The standard markers used for MW. determination were blue dextran (2,000 kD), aldolase (158 kD), catalase (240 kD), ferritin (440 kD), thyroglobulin (660 kD) and potassium dichromate (294 D). A plot of elution volume versus the log of the MW of these standard markers will give a standard curve for calibrating the unknown MW of the native protein.

13. Polyacrylamide Gel Isoelectric Focusing

Isoelectric focusing was performed in Bio-Rad minigel IEF apparatus (Model 111 Mini IEF Cell) by using the method described by Bio-rad company (mini IEF gel manual). The polyacrylamide slab gel (3.16 ml) contain 0.625 ml of monomer concentrated stock solution, comprising of 25% (w/v) acrylamide and 0.07% bis (N,N'-methylene bis-acrylamide), 0.625 ml of 25% (v/v) glycerol, 0.16 ml of Bio-lyte ampholytes 3/10 and 1.72 ml of deionized water. The gel mixture was degassed for 5 min. before it was initially polymerized by adding 10 µl of 0.02 ammonium persulfate, 15 µl of

0.1% riboflavin and 5 μl of TEMED, and immediately pored into the casting tray. The gel was photopolymerized for 45 min at room temperature. After the gel had set completely, the casting tray was removed. The purified CS-HLLBP sample and standard markers were directly applied to the gel surface and left to diffuse into the gel for 5 min. Focusing was carried out at constant voltage in a stepwised manner at 100 V for 15 min, 200 V for 15 min and finally 450 V for 60 min. After focusing was completed, the glass plate was removed from the gel supporting film. The protein bands in IEF gel were visualized by staining with solution containing 0.04% (w/v) Coomassie Brilliant blue G 250, 27% (v/v) isopropanol and 1% (v/v) acetic acid and followed by destaining with solution containing 25% (v/v) isopropanol and 7% (v/v) acetic acid until the background was clear.

The IEF markers (Kit for Isoelectric Focusing range 3.9-9.3) used for determination pI value of HLL and CS-HLLBP were amyloglucosidase (pI 3.6), trypsin inhibitor (pI 4.6), β-lactoglubulin (pI 5.1), cabonic anhydrase II (pI 5.9), cabonic anhydrase I (pI 6.6), myoglobulin (pI 6.8 and 7.2), lentil lectin (pI 8.6, 8.6, and 8.8), and trypsinogen (pI 9.3).

14. pH stability of *Hevea* latex lectin and C-serum *Hevea* latex lectin binding protein

The pH dependences of HLL and CS-HLLBP were measured by incubating the sample for 1 hour in the various buffers : 50 mM glycine-HCl buffer (pH2.6), 50 mM sodium acetate buffer (pH 4 and 5), 50 mM sodium phosphate buffer (pH 6 and 7), 50 mM Tris-HCl buffer (pH 7 and 8) and 50 mM glycine-NaOH buffer (pH 9 and 10). After the incubation the mixture pH was adjusted to 7.4 with 1 M Tris-HCl pH 7.4 and the respective H.A. or H.I. was dertermined in TBS. The results were expressed as percentages of the control sample kept under TBS as 100% H.A. or H.I. activity.

15. Thermal stability of *Hevea* latex lectin and C-serum *Hevea* latex lectin binding protein

The purified HLL and CS-HLLBP were tested for thermal stability by incubated at various temperatures at 4 (control), 25, 30, 40, 50, 60, 70, 80, ^{o}C for 30 min in a reciprocating shaking water bath. At the end of incubation, the protein aliquots were immediately placed in an ice bath and assayed for their respective H.A. or H.I. activity. The results were expressed as percentages of the control (sample kept at 4 ^{o}C) as 100% H.A. or H.I. activity.

16. Effects of strong pHs and enzymatic treatments on *Hevea* latex lectin

The effect of chitinase, pronase, extreme acid and alkaline pH treatments were performed on HLL. The purified HLL 100 µl containing 0.5 mg/ml was incubated with equal volume chitinase (5 mg/ml), pronase (5 mg/ml), 0.1 N NaOH, 0.1 N HCl and 5 % TCA. The treaments were carried out for 30 min, 60 min and 120 min at room temperature. At the end of the incubations, the mixture pH was neutralised to 7.4 and evaluated for the remaining H.A. activity.

17. Precipitin reaction.

17.1 Precipitin formation between *Hevea* latex lectin and C-serum *Hevea* latex lectin binding protein

A precipitin reaction was conducted by slightly modified method of So and Goldstein (1967). Dialyzed C-serum protein solution was prepared from protein pellet fraction obtained from ammonium sulfate fractionation between 60-80% saturation. The sample solution 100 µl containing 0.52 mg/ml, was serially diluted with 100 µl of TBS and followed by addition of 50 µl of a solution containing 26 µg of HLL giving a total precipitin reaction volume of 250 µl. After incubation for 4 hours at 37°C,

reaction mixtures were kept for 48 hours at 4°C and the resulting precipitates were collected by centrifugation at 15,000 g for 30 min. It was then washed with cold TBS and dissovled in 0.5 M NaOH. The samples were subjected to SDS-PAGE and visualized after Coomassie Brilliant blue staining.

17.2 Precipitin formation between *Hevea* latex lectin and *Hevea* latex lectin binding protein from small rubber particles

The SRP-HLLBP was extracted from washed rubber particless zone 2 with 0.2% Triton X-100, The sample solution 100 µl 0.52 mg/ml was serially diluted with 100 µl of TBS and followed by addition of a 50 µl solution containing 26 µg of HLL giving a total precipitin reaction volume of 250 µl. After incubation for 4 hours at 37 °C, the reaction mixtures were kept for 48 hours at 4°C and the resulting precipitates were collected by centrifugation at 15,000 g for 30 min. It was washed with cold TBS and dissovled in 0.5 M NaOH. The samples were subjected to SDS-PAGE and visualized after Coomassie Brilliant blue staining.

18. Protein determination

Protein concentration was determined by the method of Lowry *et al.*, (1951) using bovine serum albumin (BSA) as a standard.

The assay mixture contained 100 µl of protein sample solution and 3 ml of freshly prepared alkaline copper solution (100 ml of 2% Na_2CO_3 in 0.1 N NaOH was mixed with 1 ml of 1% potassium tartate and 1 ml of 0.5% $CuSO_4.5H_2O$). the mixture was mixed and left at room temperature for 10 minutes, then 0.3 ml of 1 M Folin phenol reagent was added and mixed thoroughly. This mixture was left standing for 30 minutes before measuring its absorbance at 650 nm in the DU 650 ; Spectrophotometer Beckman.

19. Hemaggglutination inhibition activity assay of C-serum *Hevea* latex lectin binding protein

The 50 µl of various concentration of CS-HLLBP was incubated with 50 µl of HLL for 30 min, at room temperature. At the end of incubation, the remaining hemagglutination activity of HLL was measured. An aliquot of 50 µl was taken from the mixture and serially diluted with 50 µl of TBS. Then 50 µl of 2% erythrocyte suspension was added and remaining H.A. was determined after an hour. The control test was performed in the same mamner by incubating HLL with TBS instead of CS-HLLBP. The result was expressed as remaining H.A. activity.

20. Effect of glycosidase treatments on hemagglutination inhibition activity of C-serum *Hevea* latex lectin binding protein

An aliquot containing 50 µl of CS-HLLBP (0.8 mg/ml) was treated with 50 µl of each glycosidase under specified activity unit (see Table 8). After the incubation at room temperature for 15 min the hemagglutination inhibition activity of CS-HLLBP was determined from each reaction mixture. An aliquot containing 25 µl of the incubation mixture was serially diluted into 25 µl of TBS. The diluted HLL (25 µl) containing H.A. titer of 4 was added into each well. After further incubation for 20 min, the hemagglutination was observed by adding the 2% (v/v) erythrocyte suspension. Then the hemagglutination inhibition activity was calculated. The control assay was performed in the same manner by incubating CS-HLLBP with hemagglutination assay buffer instead of glycosidase. The result was expressed as percentages of the control which calculated to be 100% activity.

21. Preparation of asialoglycoprotein

The incubation mixture contained the following components in a total volume of 0.25 ml, sodium acetate pH 5.4, 25 µmoles; bovine submaxillary mucin (BSM), 4 µmoles and neuraminidase from *Salmonella*

tryhimurium recombinant, 0.04 unit. The mixture was incubated for 60 min. at 37 °C and stopped by heating for 90 sec at 100 °C.

22. Rubber particles aggregation assay

A new method was developed to detect rubber particles aggregation induced by HLL. This method employed the rubber particles (Zone 2) that had been washed several times with TBS and diluted with the same buffer to reach absorbance of 0.8-0.9 at 600 nm. The rubber particles suspension (25 µl) was incubated with 25 µl of various protein samples containing either C-serum, B-serum , BSA or HLL, for 30 min. The mixture was stained by mixing with 5 µl of 10 % (w/v) basic Fuchsin. The stained mixture was withdrawn into hematocrit tube and the withdrawal tip was plugged with crayon. The stained rubber aggregate was separated into the top layer of the tube by centrifugation for 5 min in a hematocrit centrifuge. The rubber aggregates were examined under a microscope. The aggregation control assay was done by mixing rubber particles with TBS.

23. Rubber particles aggregation inhibition assay.

HLL (15 µl) containing 5 µg of protein was preincubated with 15 µl of CS-HLLBP containing various protein amounts ranging between

8.13-130 μg as indicated. After the preincubation period for 30 min, 30 μl washed rubber particless were added to the mixtures and mixed well. The incubation was carried out for 30 min and the mixture was stained by mixing with 5 μl of 10%(w/v) basic Fuchsin. The mixture was then withdrawn into a hematocrit tube and followed by plugging the drawing tip with crayon. The rubber aggregate was separated into the top layer of the tube by centrifugation for 5 min in a hematocrit centrifuge. The rubber aggregation was then examined under a microscope. The aggregation control assay was done by mixing rubber particless with TBS instead of a mixture containing HLL and CS-HLLBP.

24. Rubber particles aggregation by commercial lectins

Lectins purchased from Sigma were used to test for rubber particles aggregation. The rubber particles (Zone 2) which were washed several times with TBS and then diluted to attain 0.8-0.9 absorbance at 600 nm in the same buffer. This diluted rubber particles solution (25 μg) was preincubated with 25 μl (1 mg/ml) of the following lectins : Concanavalin A , *Erythrina cristagalli*, *Maackia amurensis*, *Sambucus nigra*, *Cicer arietinum* or HLL (0.58 mg/ml) for 30 min. After the preincubation period for 30 min 30 μl washed rubber particles were added to the mixtures and

mixed well. The incubation was carried out for 30 min and the mixture was stained by mixing with 5 µl of 10%(w/v) basic Fuchsin. The mixture was then withdrawn into a hematocrit tube and followed by plugging drawing with crayon. The rubber aggregate was separated into the top layer of the tube by centrifugation for 5 min in a hematocrit centrifuge. The rubber aggregation was then examined under a microscope. The aggregation control assay was done by mixing rubber particles with TBS instead of lectin.

25. Correlation between activity levels C-serum *Hevea* latex lectin binding protein and rubber yield per tapping

Nine rubber trees giving high, medium and low levels of rubber yield per tapping were used for the correlation study. The fresh latex from each tree was collected in the plastic containers which were chilled on ice. The C-serum was separately prepared from each latex sample by ultracentrifugation 59,000 g for 45 min. The levels of H.I. due to the presence of CS-HLLBP were determined from respective C-serum and expressed as the total hemagglutination inhibition activity per tapping. A correlation curve between levels of CS-HLLBP activity and rubber yield per tapping was constructed.

Chapter 3

Results

When the latex vessels are cut open by tapping their expelled contents can be collected for a certain length of time before cessation takes place. The amount of rubber latex yield is directly proportional to the flowing time. The cessation of the latex flow is carried out by latex vessel plugging process involving development of plugs or flocs to seal off the exuding vessels at or very near the vessels cut ends (Pakianathan *et al.*, 1966). This biochemical process leading to latex vessel plugging is necessary for the tree to minimize the loss of its metabolite products. The flocs and caps formed at the open extremity of latex vessel had been examined by electron microscope. It was found to consist of lutoid membrane debris and rubber particles (Southorn, 1968). The bursting of lutoid particles happening during the latex flow could be due to various factors such as mechanical shearing, exposure to the atmospheric gas pressure as well as osmotic change within the latex vessel. It was also shown that the higher the lutoid bursting index, the faster the coagulum formation. The lutoid particles was suggested to play a key role in rubber latex coagulation with subsequent plugging of the latex vessels.

At present, the mechanism involved coagulum formation is not clearly understood. Hevein was previously shown to have specific recognition on rubber particles protein and suggested to aggregate rubber particles in a lectin-like manner (Gidrol *et al.*, 1994). However, the nature of rubber particles aggregates induced by soluble (lectin-like) hevein is different from natural rubber coagulum found at the vessel end which composed of lutoid membrane debris as well as rubber particles.

In order to elucidate the mechanism on coagulum formation, the scope of study in this thesis is aimed to identify and isolate a membrane bound lectin from lutoid particles and demonstrate its aggregate binding capacity with rubber particles. In additions, an intrinsic latex soluble lectin binding protein in the serum phase of latex (C-serum) is also identified and its function suggested.

Accordingly, the first part of the experimental results was dealt with isolation and purification of lectin from lutoid membrane. This protein was called *Hevea* latex lectin and designated as HLL. The second part was on characterization of purified HLL. The third part involved isolation and purification of C-serum *Hevea* latex lectin binding protein, designated as CS-HLLBP. The fourth part covered characterization of purified CS-HLLBP. The final part related to function verifications of HLL as an coagulating factor in inducing rubber particles aggregation leading to latex vessels

plugging and CS-HLLBP as an anti-coagulating factor in preventing HLL-induced rubber particles aggregation or promoting latex flow duration.

Part 1. Purification of *Hevea* latex lectin

1.1 Preparation of lutoid membrane.

Fresh latex obtained from tapping was separated into 3 layers by ultracentrifugation. The bottom fraction, containing membrane bound organelles called lutoid particles, was isolated and repetitively washed with TBS. The lutoid membrane was isolated from bursted lutoids obtained upon using acetone and distilled water, as shown under Method A and B of Fig. 12, respectively.

1.2 Extraction and purification of *Hevea* latex lectin

A non-ionic detergent was employed to release the tightly bound HLL from the lutoid membrane. The resulting protein extract was found to be able to agglutinate erythrocytes from rabbits and mice but not human. According to Method A (Fig. 12), the HLL was extracted from washed lutoid membrane in the presence of a non-ionic detergent 0.2% Triton-X 100. The extract was purified through chitin batch-binding and DEAE-Sepharose column and the data shown in Table 2. The final purification fold increased 9.9 times and specific activity was 204,800 titer/mg. Under this Method we obtained the 0.2 mg of purified HLL from 350 ml of fresh latex. Under the Method B, the HLL was extracted twice with 0.5 and 0.2% Triton X-100, respectively. The first extraction was performed on washed lutoid membrane and separated into an upper phase layer by introducing phase separation with ammonium sulfate at 40%

saturation. The extract was concentrated by acetone precipitation and re-extracted in 0.2% Triton X-100. The extract was purified through a single ion-exchange chromatography step by using DEAE-Sepharose column and data shown in Table 3. Accordingly, Method B gave slightly lower value in specific activity (163,840 titer/mg) as compare to Method A (204,800 titer/mg). However, Method B seems to be more suitable for large-scale preparation since the yield on purifed HLL per unit volume of fresh latex is 5 μg/ml and about 8.8 fold higher than that obtained under Method A. A comparable level of specific activity was obtained under both methods but with slightly lower value under Method B.

The purified HLL samples obtained from final purification steps under Method A and B were analyzed by SDS-PAGE. Under Method A a single protein band of HLL with molecular weight 17 kD was observed as shown in Fig. 19.1-19.2. Under Method B, a prominent band of purified HLL, as see under Method A, was observed together with slight amount of other higher molecular weight proteins. This result suggests an additional further requirement on chitin affinity purification in order to achieve homogeneity purification of HLL, similar to that obtained under Method A.

Table 2 The purification of HLL under Method A

Step	Total protein (mg)	H.A. (titer)	Specific activity (titer/mg)	Yield (%)	Purification (fold)
HLL extract*	15.8	819,200	20,739	100	1
Chitin batch-binding	0.29	184,320	80,139	22.5	3.9
DEAE-Sepharose	0.20	266,240	204,800	32.5	9.9

*The extraction was done in the presence of 0.2% Triton X-100 on acetone precipitated fraction obtained from lutoid membrane isolated from bottom fraction of ultracentrifuged fresh latex (350 ml).

Table 3 The purification of HLL under Method B

Step	Total protein (mg)	H.A. (titer)	Specific activity (titer/mg)	Yield (%)	Purification (fold)
HLL extract *	24	1,638,400	68,267	100	1
DEAE-Sepharose	5	819,200	163,840	50	2.4

*The extraction was done in the presence of 0.2% Triton X-100 on acetone precipitated fraction obtained from lutoid membrane isolated from bottom fraction of ultracentrifuged fresh latex (1,000 ml).

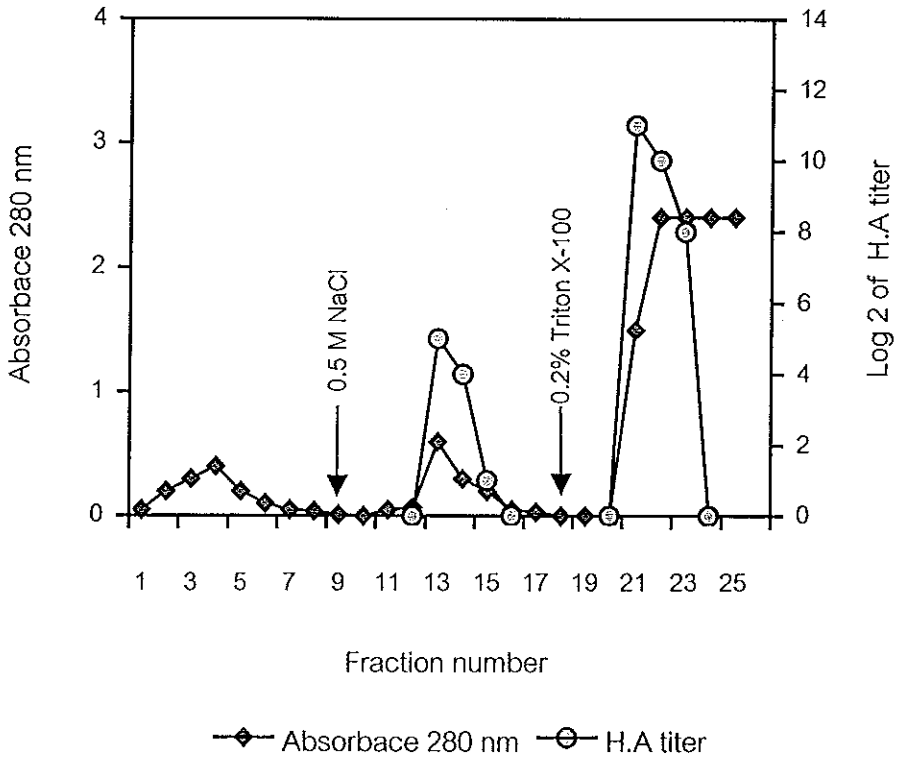


Fig.15 Elution profile of HLL on a DEAE-Sepharose column chromatography

HLL extract solution obtained from Method A was loaded onto a DEAE-Sepharose column (1.5 x5 cm) preequilibrated with 50 mM Tris-HCl pH 7.4 at a flow rate of 15 ml/h and washed with the same buffer until the effluent absorbance at 280 nm approached zero. The column was stepwise eluted with 0.5 M NaCl and followed by 0.2% Triton X-100 in the same buffer, respectively. Each fraction was assayed for HLL activity and fraction exhibited hemagglutination activities were pooled and used for further characterization.

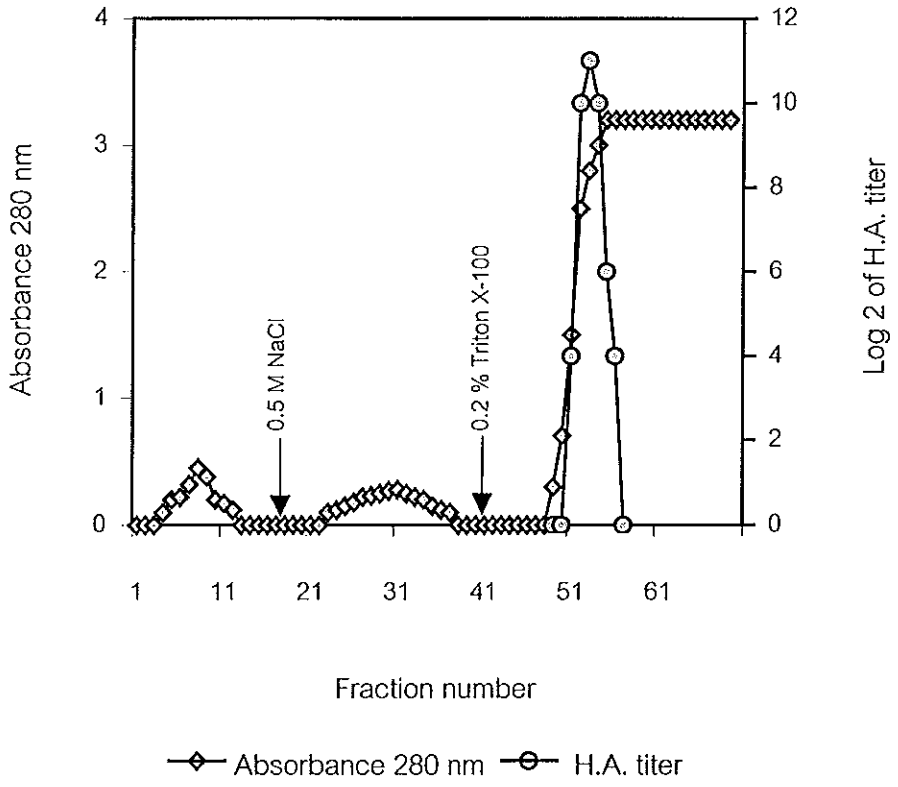


Fig.16 Elution profile of HLL extract on a DEAE-Sepharose column chromatography

HLL extract solution obtained from Method B was loaded onto a DEAE-Sepharose column (1.5 x 12 cm) preequilibrated with 50 mM Tris-HCl pH 7.4 at a flow rate of 15 ml/h and washed with the same buffer until the effluent absorbance at 280 nm approached zero. The column was stepwise eluted with 0.5 M NaCl and followed by 0.2% Triton X-100 in the same buffer, respectively. Each fraction was assayed for HLL activity and fraction exhibited hemagglutination activities were pooled and used for further characterization.

1.3 Carbohydrates/glycoproteins binding specificity of *Hevea* latex lectin

The carbohydrate binding specificty of HLL to either free sugars or sugars as protein conjugated form could be determined from its inhibitory effect on hemagglutination activity of HLL. This can be expressed as 100% H.I. activity at various levels of the glycoprotein with positive effect and the specific binding of these glycoprotein reflected the sugars specificity of the HLL. Several sugars and glycoproteins including CS-HLLBP and SRP-HLLBP were used in these experiments and their activity on hemagglutination inhibitons were evaluated.

According to Table 4, the HLL-binding was more specific to glycoproteins originated from latex namely CS-HLLBP and SRP-HLLBP than any other sources. Its binding specificity obtained with CS-HLLBP is 10 times higher than that of SRP-HLLBP giving with 100% H.I. activity at 0.02 mg/ml and 2 mg/ml, respectively. The result showed specific binding of HLL with all glycoproteins studied except for α_1-acid glycoprotein.

Furthermore, the glycoproteins containing sialic acid (fetuin and mucin) showed a stronger binding specificities with HLL than those containing no sialic acid (asialofetuin and asialomucin). Similary, an inhibitory effect on H.I. activity was obtained with prior treatment of bovine submaxillary mucin (BSM) with neuraminidase (Fig 17). The inhibitory

activity of 0.3 mg/ml BSM was decreased by 50% and 25% on incubation with neuraminidase for 5 and 15 min at 37 °C, respectively.

Among various free carbohydrates studied (Table 5) non of the mono, di or tri saccharides, at rather high concentrations as indicated, could inhibit hemagglutination induced by HLL.

Table 4 Glycoprotein binding specificity of HLL

Glycoproteins	100% Hemagglutination inhibition * (mg/ml)
Fetuin	0.625
Asialofetuin	1.25
Mucin	0.625
Asialomucin	2.5
Ovomucoid	2.5
Trypsin inhibitor (type II-S)	1.25
SRP-HLLBP	0.20
CS-HLLBP	0.02
α_1-acid glycoprotein	**

* Concentration required for 100% inhibition of agglutination of rabbit erythrocytes in the presence of HLL (1.17 μg/ml)

** No hemagglutination inhibition observed at 5 mg/ml.

Table 5 Non-effective carbohydrates observed in binding specificity study of HLL

Carbohydrate	Concentration*
Glucose, Galactose, Mannose, Fucose	200 mM
Arabinose, Melibiose, Lactose, Glucosamine,	
Raffinose, GlcNAc, ManNAc, GalNAc	100 mM
Chitosan dimer, Chitosan trimer	5 mg/ml
Gal 1→ 3 GlcNAc, Gal 1→ 4 GlcNAc	
Gal 1→ 6 GlcNAc	2 mg/ml
3'NeuNAc-Lac, 6'NeuNAc-Lac	2 mg/ml
NANA	5 mg/ml

*Concentration of each sugar used for the H.I. test in the presence of HLL (1.17 μg/ml)

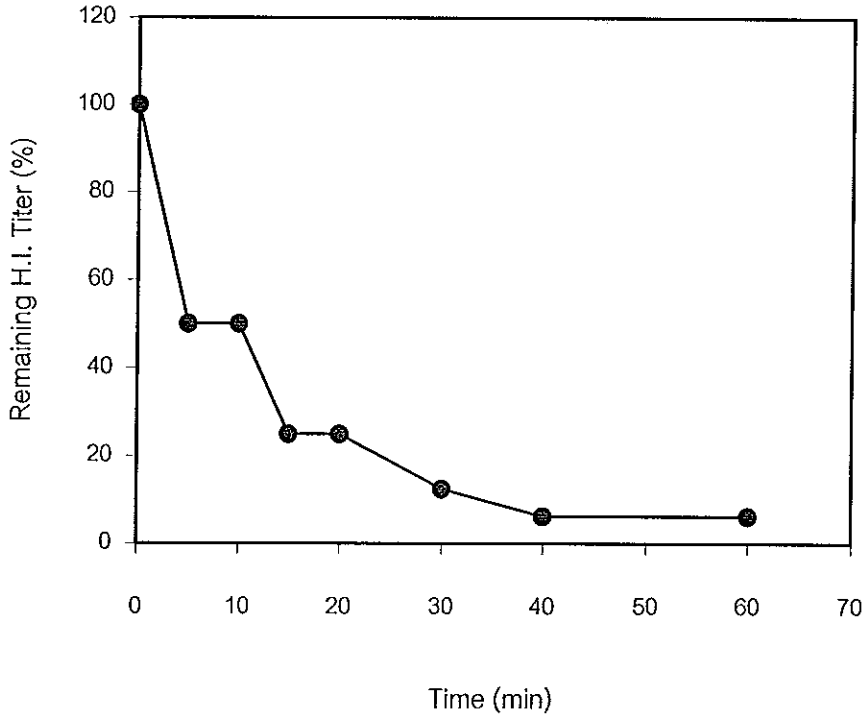


Fig. 17 Time course on inhibitory effect of BSM in hemagglutination assay upon neuraminidase treatment

The 0.3 mg/ml of bovine submaxillary mucin (BSM) was treated with 0.04 unit of neuraminidase from *Salmonella typhimurium* recombinant. The mixtures were incubated from 0 to 60 min at 37 °C and stopped by heating for 90 sec. at 100 °C. After that, BSM was tested on its ability to inhibit hemagglutination activity of HLL. The control with 100% H.I of BSM was done in the same way but sodium accetate buffer was used instead of enzyme.

Part 2. Characterization of *Hevea* latex lectin

2.1 Native molecular weight determination of *Hevea* latex lectin

For the determination of native molecular weight of HLL, the purified HLL obtained from DEAE-Sepharose column and standard marker proteins were subjected to gel filtration on Sepharose CL 6B column (2.5x 85 cm) in 50 mM Tris-HCl buffer containing 0.1% Triton X-100. A plot between respective values on the K_{av} of standard proteins and logarithm of their molecular weight was constructed. The native molecular weight of purified HLL obtained from Sepharose CL 6B column was calibrated to be 412 kD (Fig. 18).

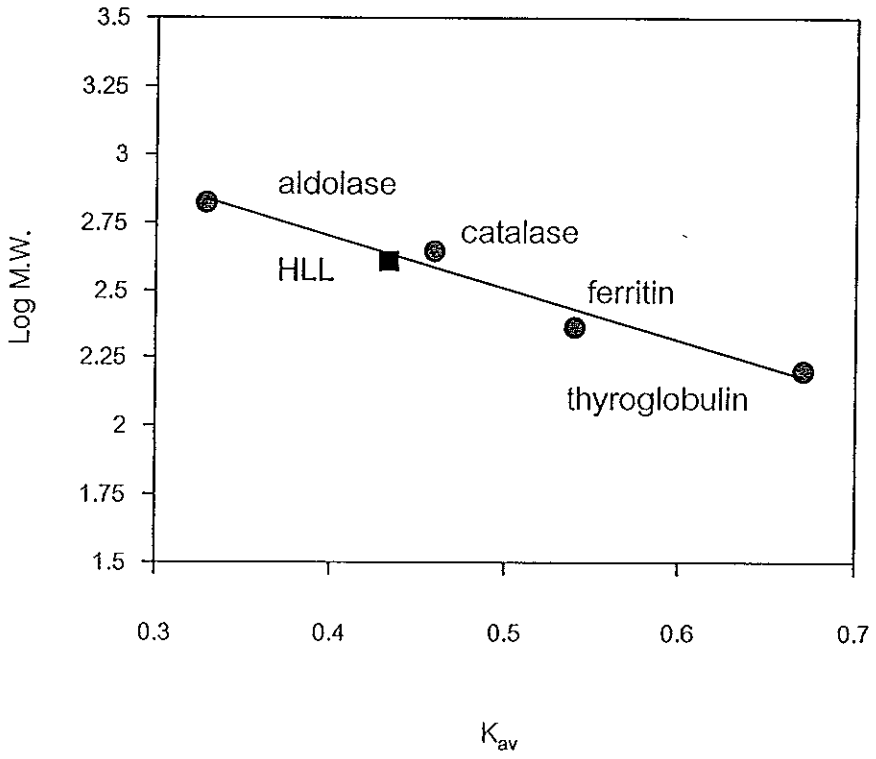


Fig. 18 Molecular weight calibration of HLL by gel flitraion on Sepharose CL 6B column.

The calibration curve was plotted between the respective values on the K_{av} of standard protein and logarithm of their molecular weights. The standard protein were aldolase (158 kD), catalase (232 kD), ferritin (440 kD) and thyroglobulin (660 kD). The molecular weight of HLL was calibrated to be 412 kD, according to the value obtained at the interception.

2.2 Determination of subunit molecular weight of *Hevea* latex lectin

For determination of subunit molecular weight of HLL, the purified HLL obtained from DEAE-sepharose column chromatography and Pharmacia standard marker proteins were subjected to SDS-PAGE on a 12% slab gel. The result shown in Fig. 19.1-19.2 indicated that HLL purification reached homogeniety under Method A but not B (Fig. 20). The purified HLL is composed of homomeric protein subunit, calibrating to be 17 kD (Fig. 21). The result suggests that the native HLL of 412 kD, is a multimeric protein and composed of roughly 24 homomeric subunits.

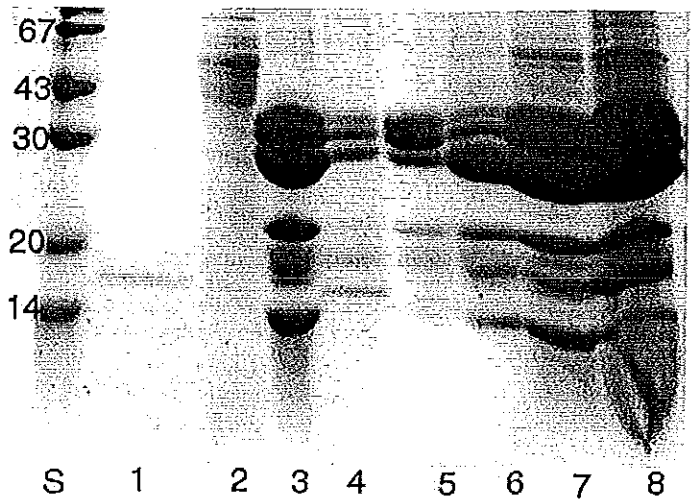


Fig. 19.1 SDS-PAGE analysis of purified HLL under Method A, Fig. 12 (Prep. No. 1)

S = standard protein markers;

1 = eluted purified HLL fraction from DEAE-Sepharose column @ 0.2% Triton X-100;

2 = eluted protein fraction from DEAE-Sepharose column @ 0.5 M NaCl;

3 = unbound fraction from DEAE-Sepharose column;

4,5 = eluted protein fraction from chitin batch-binding @ 0.5 M NaCl and 0.2% Triton X-100, respectively;

6,7 = unbound fraction from chitin batch-binding;

8 = HLL extract.

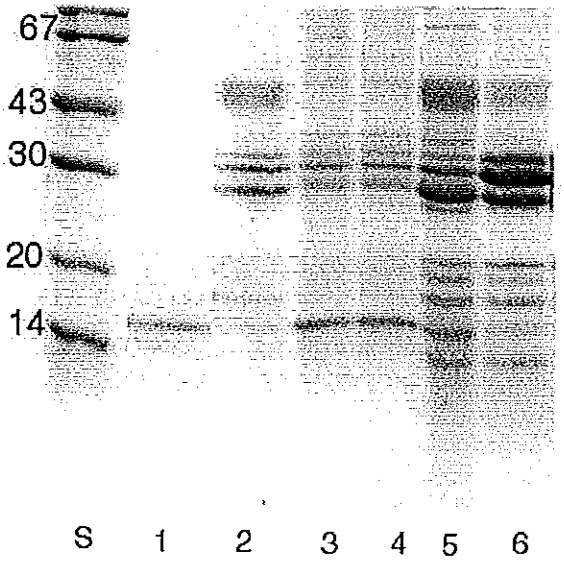


Fig. 19.2 SDS-PAGE analysis of purified HLL under Method A, Fig 12. (Prep. No. 2)

S = standard protein markers;

1 = eluted protein fraction from DEAE-Sepharose column with 0.2% Triton X-100;

2 = eluted protein fraction from DEAE-Sepharose column with 0.5 M NaCl;

3,4 = eluted protein fraction from chitin batch-binding with 0.2% Triton X-100;

5 = unbound fraction from chitin batch-binding;

6 = HLL extract.

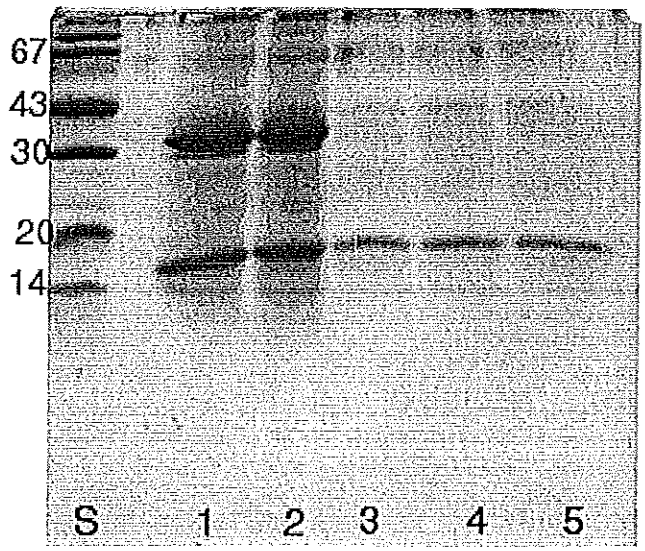


Fig. 20 SDS-PAGE analysis of purified HLL under Method B

S = standard protein markers;

1,2 = 2 [nd] HLL extract (from the acetone pellet fraction);

3,4 = eluted HLL fraction from DEAE-Sepharose column with 0.2% Triton X-100.

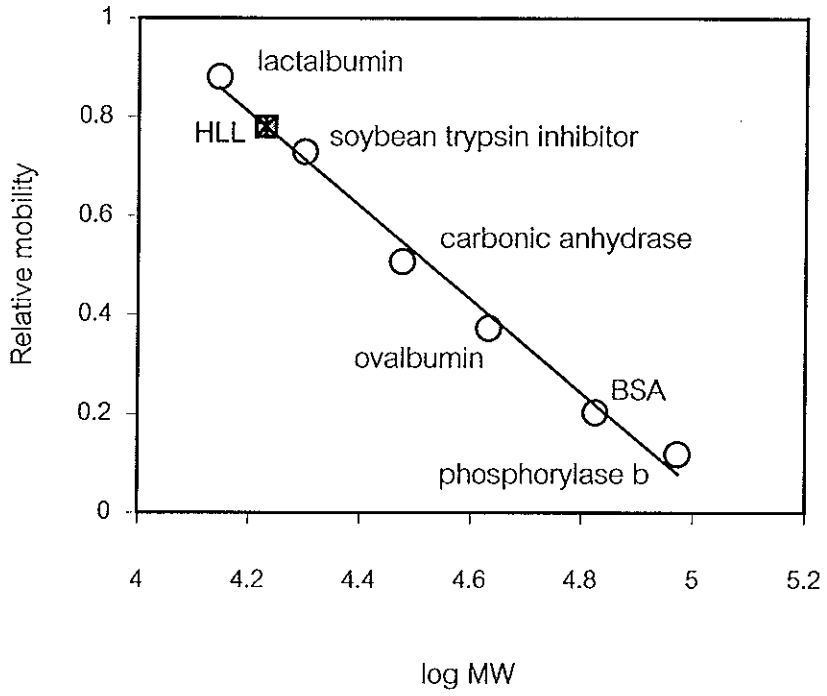


Fig. 21 Subunit molecular weight calibration of HLL on SDS-PAGE

The calibration curve was plotted between relative mobility of the standard proteins and the logarithm of their corresponding molecular weights. The standard protein references were α-lactalbumin (14 kD), soybean trypsin inhibitor (20 kD), carbonic anhydrase (30 kD), ovalbumin (43 kD), bovine serum albumin (67 kD) and phosphorylase b (94 kD).

2.3 Thermal stability of *Hevea* latex lectin

To test for the heat stability, HLL was subjected to incubation at different temperatures before measuring its remaining activity. The HLL was heated for 30 min at various temperature ranging from 4 °C to 80 °C as indicated (Fig. 22). The remaining H.A. activity was expressed as a percentage of the control, treated at 4 °C containing 100% H.A. activity. There is no lost in H.A. activity after heating upto 60 °C for 30 min. A sharp decrease in its activity was, however, observed when temperature raised from 70 °C to 80 °C where half of its activity still retained. This indicated that HLL is a heat stable protein similar to *Hevea* bark lectin (Wititsuwannakul, 1998).

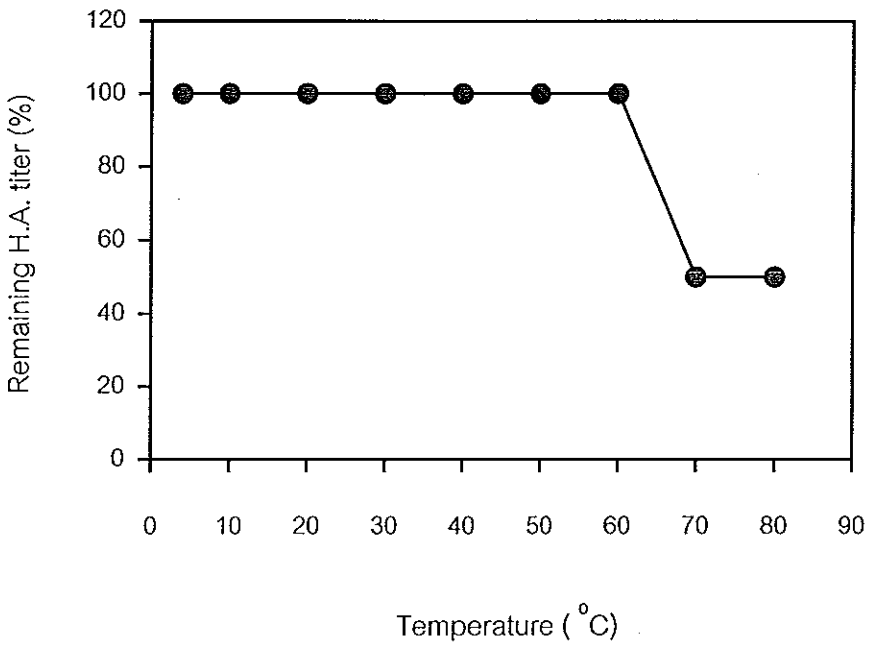


Fig. 22 Thermal stability of HLL

The purified HLL eluted from DEAE-Sepharose column was incubated at various temperature as indicated for 30 min and the remaining hemagglutination activity measured. The result was expressed as the percentage of remaining activity as compared to the control, kept at 4 °C.

2.4 pH stability of *Hevea* latex lectin

The effect of pH on the hemagglutination activity of HLL was performed by incubating HLL at various pH ranges of 4-10 for 1 hour as described in the Methods. The HLL solution was then adjusted back to pH 7.4 before measuring the remaining H.A. activity and expressed as percentage of the control activity. The result showed that HLL is quite stable over a wide pH range from 5-10 whereas one-half of its activity disappeared at pH 4. (Fig 23). This indicates that the optimum pH stability of HLL is between 5-10 with full activity. A lost on half its activity at pH 4 might reflects limitation on pH dependent of the subunit association in forming active HLL.

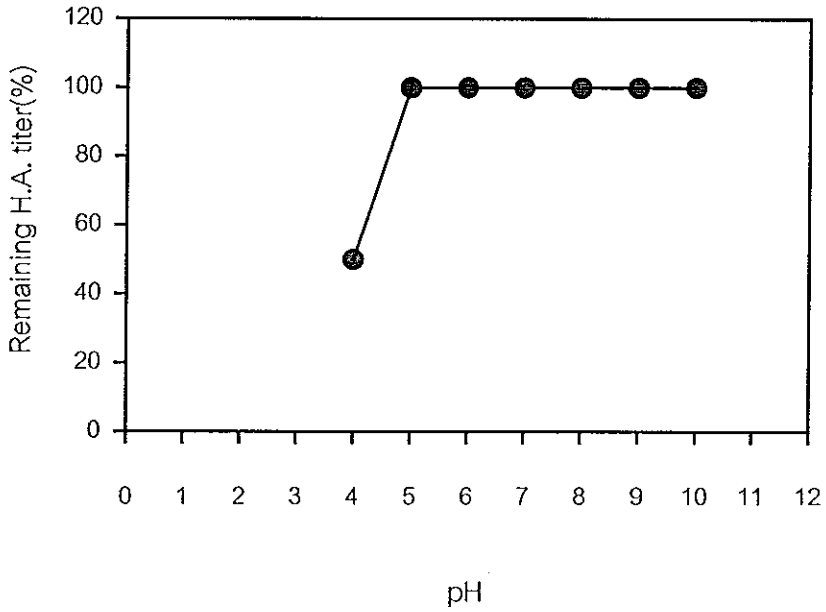


Fig. 23 pH stability of HLL

The purified HLL eluted from DEAE-Sepharose column was incubated at various pHs, from 4 to 10 as indicated for 1 hour. The pH of samples were then readjusted back to 7.4. The remaining hemagglutination activity were determined and expressed as the percentage of the control kept at pH 7.4 at all time.

2.5 Effects of strong pH conditions and enzymatic treatments on activity of *Hevea* latex lectin

The previous study on pH stability of HLL revealed that it is stable under both mild acid and alkaline treatment with pHs ranging from 5-10. In extension to this study, the effect of strong acid and alkaline hydrolysis on HLL was carried out. It is seen in Table 6 that HLL is extremely stable in alkaline but acid-labile. Full HLL activity was retained after strong alkaline treatment with 0.1 N NaOH for 2 hours while no activity was recovered under acid treatment with 0.1 N HCl. In addition, the study on enzymatic treatment on HLL revealed that it is stable to pronase. This study indicates an unusual stable property of HLL to both alkaline as well as proteolytic hydrolysis.

Table 6 Effect of extreme pH conditions and proteolytic treatments on HLL activity

Treament No.	Remaining H.A. activity		
	30 min	60 min	120 min
No. 1 HLL (control)	64 (100%)	64 (100%)	64 (100%)
No. 2 = No. 1 + Pronase (5 mg/ml)	128 (200%)	128 (200%)	128 (200%)
No. 3 = No. 1 + 0.1 N NaOH	64 (100%)	64 (100%)	64 (100%)
No. 4 = No. 1 + 0.1 N HCl	0 (0%)	0 (0%)	0 (0%)

After the incubation period as indicated, the H.A. was determined in the presence of normal rabbit erythrocytes (Treatment No.1, 3 and 4) and pronase-treated rabbit erythrocytes (Treatment No. 2)

2.6 Isoelectric focusing of *Hevea* latex lectin

The IEF was carried out in this experiment for determination the pI value of HLL by using the HLL sample from the peak fraction of DEAE-Sepharose column, as described in the Method. An amount of 0.5 μg of HLL was applied on the polyacrylamide gel isoelectric focusing. After the focusing was completed, the gel containing both standard protein markers and HLL were removed from the supporting film, stained for 1 hour and followed by destaining solution until the background was at minimum in order to visualized the protein staining pattern of the IEF gel. The relative pI of the HLL band was estimated from the standard protein markers band. The band position of HLL is very close to that of myoglobulin which served as standard pI of 6.8 and 7.2 Hence, the pI of HLL was estimated to be about 7.2 .

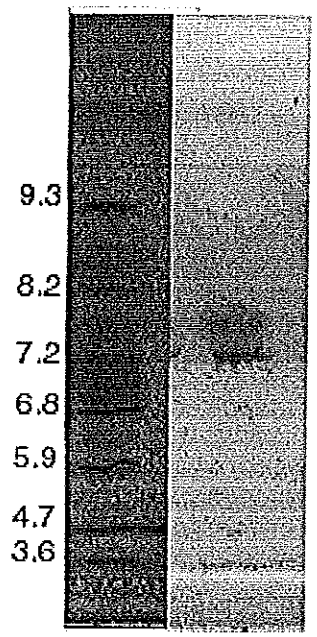


Fig. 24 pI determination of HLL

S = standard marker pI 3.6-9.3

1 = purified HLL

Part 3. C-serum *Hevea* latex lectin binding protein

3.1 Preparation and purification of C-serum *Hevea* latex lectin binding protein

A protein was prepared from C-serum fraction of ultra-centrifuged fresh latex. It was found to bind HLL by exhibiting its ability in inhibiting hemagglutination activity of HLL. The initial purification step of CS-HLLBP involved sequential fractionations by employing ammonium sulfate and acetone. Gel filtration on Bio-Gel P-300 (Fig. 25) and ion exchange chromatography on DEAE-Sephacel (Fig. 26) were respectively followed. The data on purification was shown in Table 7. The crude C-serum protein precipitated between 70-85% of ammonium sulfate was found to be a major fraction that could inhibit HLL activity. The solubilized active salt fraction was concentrated by means of acetone precipitation at 70% saturation and further purified by Bio-Gel P-300 and DEAE-Sephacel columns. The specific hemagglutination inhibition activity of purified CS-HLLBP was measured to be 4,266 titer/mg with 13.7 purification folds. A considerably high recovery yield of 33% was achieved for purified CS-HLLBP.

Table 7 Purification of CS-HLLBP

Step	Total protein (mg)	Total activity (titer)	Specific activity (titer/mg)	Yield (%)	Purification (fold)
70-85% $(NH_4)_2SO_4$ fraction	24.6	7,680	312.2	100	1
0-70% Acetone fraction	26.6	8,960	366.8	116	1.08
Bio Gel P-300	1.5	3,200	2064.5	42	6.6
DEAE-Sephacel	0.6	2,560	4266.7	33	13.7

Protein fraction obtained after ammonium sulfate precipitation of C-serum fraction isolated from of ultracentrifuged fresh latex (600 ml).

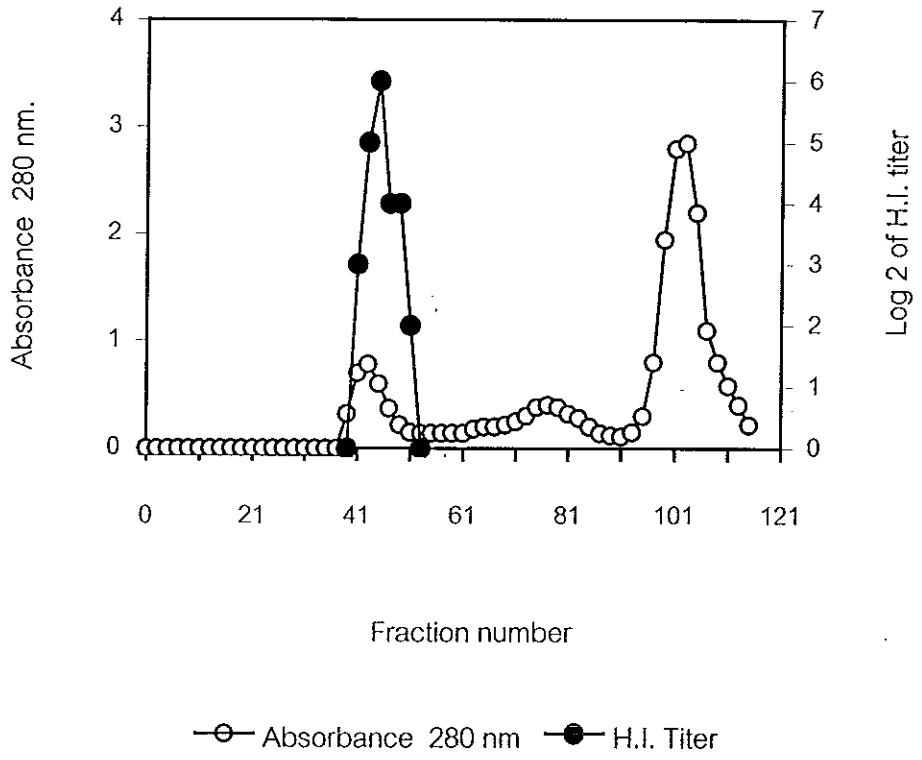


Fig. 25 Chromatographic profile of acetone-precipitated fraction of CS-HLLBP on a Bio-Gel P-300 column.

A pellet fraction obtained after acetone precipitation of protein obtained after 70-85% $(NH_4)_2SO_4$ fractionation was solubilized and loaded onto a Bio-Gel P-300 column (1.8x80 cm), previously preequilibrated with 50 mM Tris-HCl pH 7.4 at a flow rate of 15 ml/h. The column was eluted with the same buffer and 2 ml fractions were collected. The hemagglutination inhibition activity were screened on each fraction. Fractions exhibiting hemagglutination inhibition activities were pooled and concentrated for further purification step.

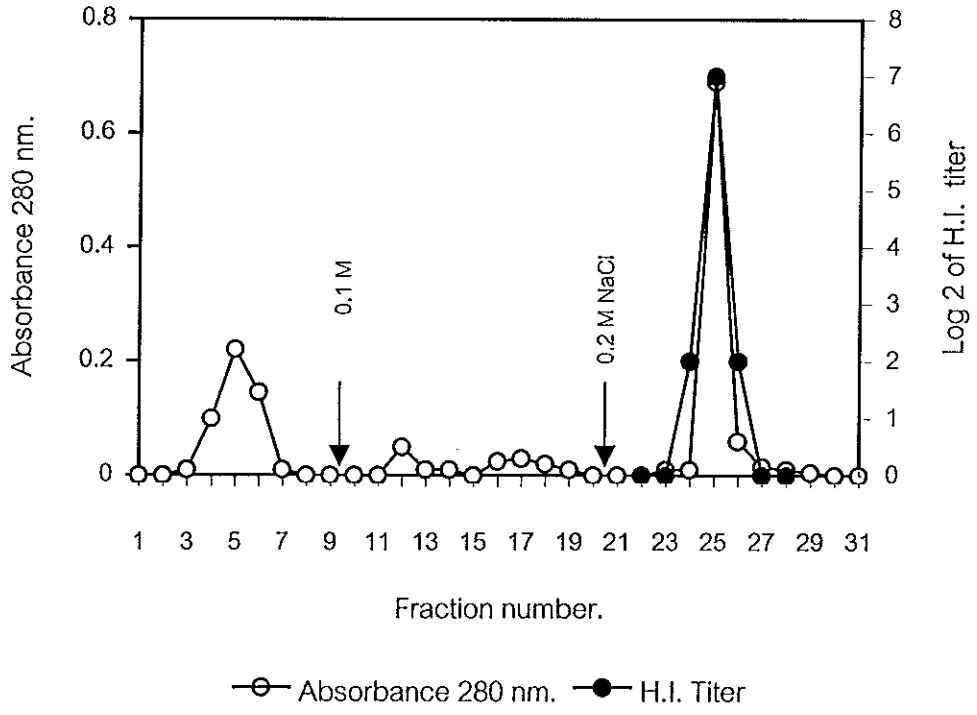


Fig. 26 Chromatographic profile of CS-HLLBP on a DEAE-Sephacel column.

The pooled activity peak fractions from Bio-Gel P-300 column were concentrated and then loaded onto a DEAE-Sephacel column (1.8 x 8 cm) previously preequilibrated with 50 mM Tris-HCl pH 7.4. at a flow rate of 15 ml/h. It was then washed with the same buffer until the effluent absorbance at 280 nm approached zero. Stepwised elution with the same buffer but containing 0.1 and 0.2 M NaCl were followed, respectively. Fractions (2ml) were collected and assayed for CS-HLLBP activity by measuring the presence of hemagglutination inhibition activity. The fractions exhibiting hemagglutination inhibition activities were pooled and used for futher characterization.

Part 4. Characterization of C-serum *Hevea* latex lectin binding protein

4.1 Native molecular weight determination of C-serum *Hevea* latex lectin binding protein

The purified CS-HLLBP, obtained from DEAE-Sephacel column and commercial standard marker proteins, were sujected to gel filtration on Sepharose CL-6B column (2.5x 85 cm) by using 50 mM Tris-HCl buffer pH 7.4. The plot between respective values on the K_{av} of standard proteins and logarithm of their molecular weights was constructed (Fig. 27). The native molecular weight of CS-HLLBP was calibrated to be 240 kD.

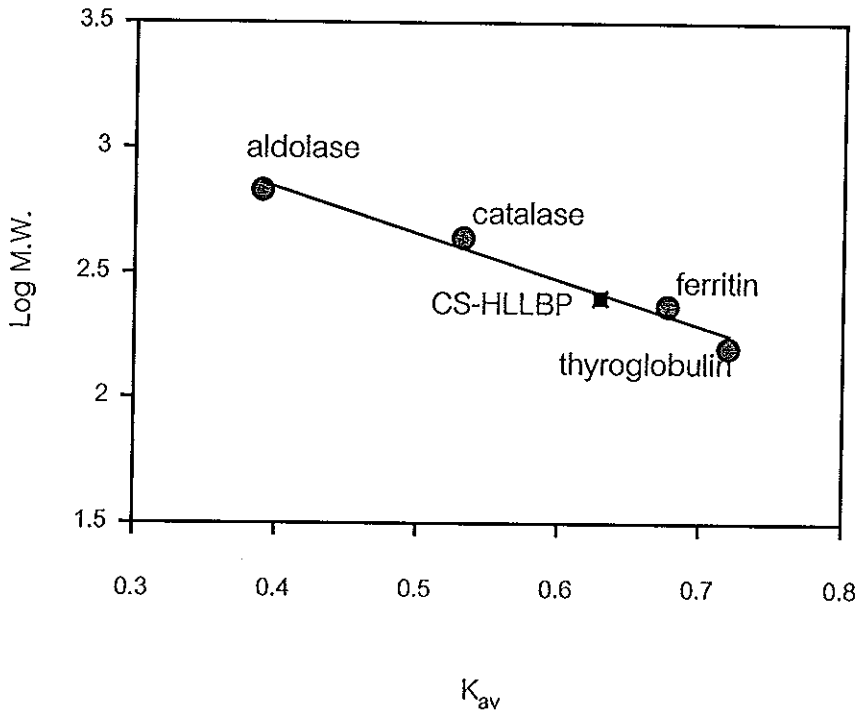


Fig. 27 Molecular weight calibration of CS-HLLBP by gel flitraion on Sepharose CL 6B column.

The calibration curve was plotted between respective values on the K_{av} of standard proteins and logarithm of their molecular weights. The standard proteins empolyed were aldolase (158 kD), catalase (232 kD), ferritin (440 kD) and thyroglobulin (660 kD). The molecular weight of CS-HLLBP was calibrated to be 240 kD, according to the value obtained at interception.

4.2 Determination of subunit molecular weight of C-serum *Hevea* latex lectin binding protein

For determination of subunit molecular weight of CS-HLLBP, the purified CS-HLLBP obtained from DEAE-Sephacel column chromatography and Pharmacia standard marker proteins were sujected to SDS-PAGE on 12% gel slab gel. The result, as shown in Fig 28 indicated that the purified CS-HLLBP is composed of multi-homomeric protein subunits seen as a single protein band. Its subunit molecular weight was estimated from a calibration curve presented in Fig. 28 to be 40 kD. Hence, the native CS-HLLBP of 240 kD. is composed of 6 homomeric subunits.

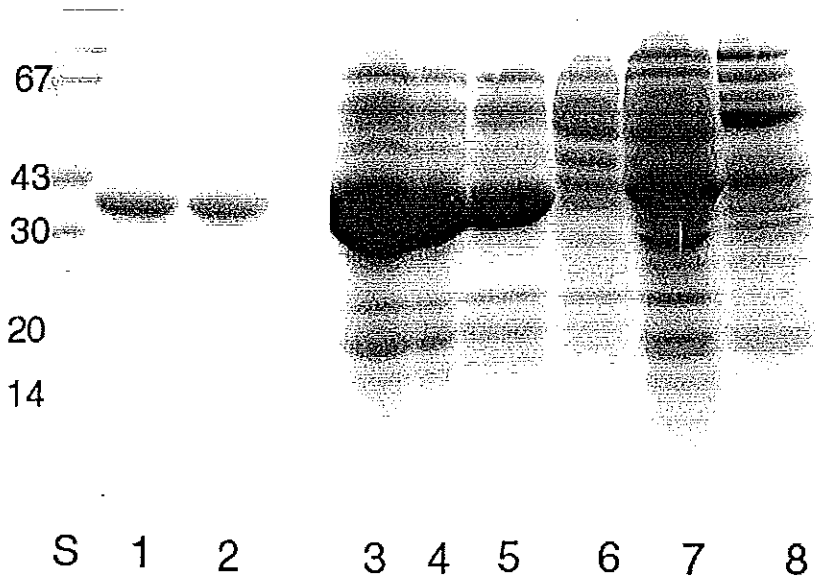


Fig. 28 SDS-PAGE analysis of purified CS-HLLBP

S = standard markers;

1,2 = eluted peak fractions @ 0.2 M NaCl from DEAE-Sephacel column;

3-5 = pooled peak fraction from Bio Gel P-300 column with loaded amount of 25, 20, 10 μl, respectively;

6-8 = protein solubilized from 40-70%, 70-85%, 0-40% $(NH_4)_2SO_4$ pellet fractions, respectively.

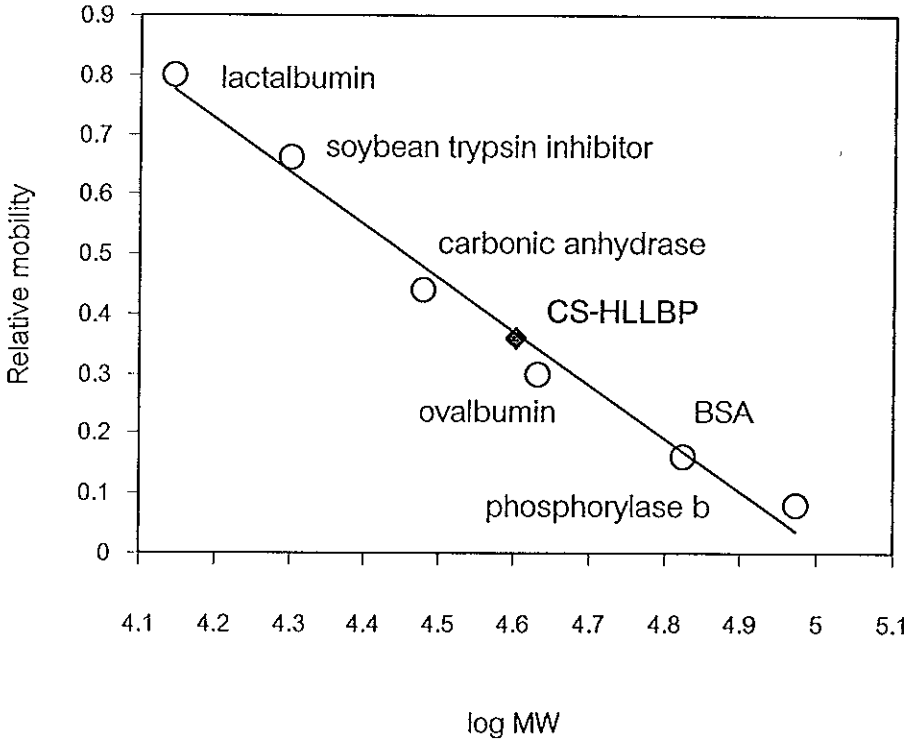


Fig. 29 Subunit molecular weight determination of purified CS-HLLBP on SDS-PAGE

The calibration curve was plotted between relative mobility of the standard proteins and the logarithm of their corresponding molecular weights. The standard proteins references were α-lactalbumin (14 kD), soybean trypsin inhibitor (20 kD), carbonic anhydrase (30 kD), ovalbumin (43 kD), bovine serum albumin (67 kD) and phosphorylase b (94 kD).

4.3 Thermal stability of C-serum *Hevea* latex lectin binding protein

The effect of temperature on activity of CS-HLLBP was evaluated by preincubating purified CS-HLLBP at different temperature before measuring its remaining activity. The CS-HLLBP was heated for 30 min at various temperature ranging from 25 °C to 80 °C as indicated (Fig. 30). The remaining H.I. activity was expressed as a percentage of the control kept at 4 °C, containing 100 % H.I. activty. Full activity of CS-HLLBP was retained after heating upto 50 °C for 30 min. A sharp decrease in its activity was observed when the temperature was raised from 60 °C to 80 °C and about 50% of CS-HLLBP activity retained. This result indicated that CS-HLLBP is relatively heat stable.

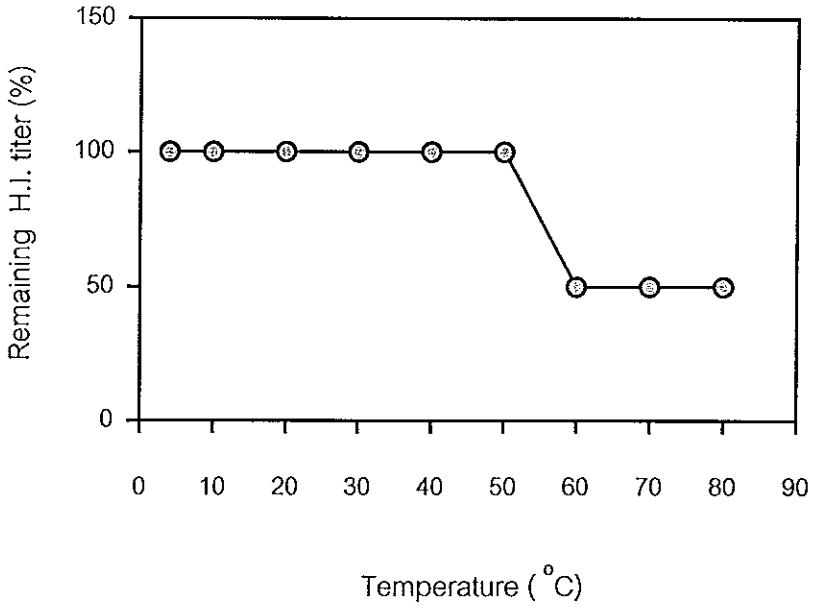


Fig. 30 Thermal stability of CS-HLLBP

The purified CS-HLLBP obtained from eluted DEAE-Sephacel column was incubated at various temperature as indicated for 30 min before measuring for the remaining hemagglutination inhibition activity. The result was expressed as the remaining activity percentage as compared to the control.

4.4 pH stability of C-serum *Hevea* latex lectin binding protein

The effect of pH on the ability of CS-HLLBP to inhibit hemagglutination of HLL was performed by preincubating CS-HLLBP at various pH ranging of 4-10 for 1 hour and pH of the solution was adjusted back to pH 7.4 before the hemagglutination inhibition assay (see Method). The results were expressed as percentage of the control where the pH was always maintained at 7.4. The result revealed that the hemagglutination inhibition activity was fully retained at pH 6-10 as shown in Fig. 31. This suggests a wide range for optimum pH stability of CS-HLLBP from 6-10. Under more acidic condition between pH 4-5, the protein lost half of its activity. This may reflect limitation on pH dependent of the subunit association of native CS-HLLBP.

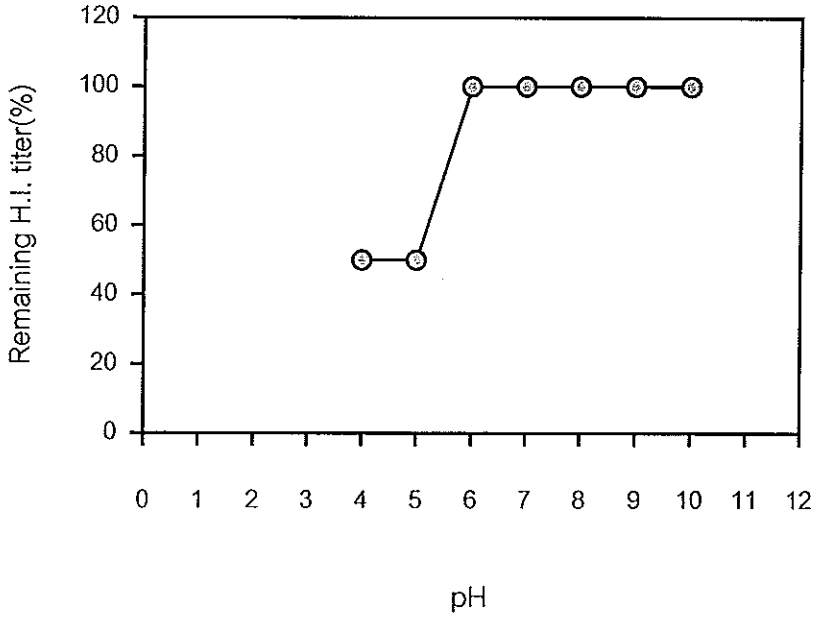


Fig. 31 pH stability of CS-HLLBP

The eluted CS-HLLBP fraction from DEAE-Sephacel column was incubated for 1 hour at various pHs from 4 to 10 as indicated. The sample pH was then readjusted back to pH 7.4. The hemagglutination inhibition activity was determined and expressed as the percentage of remaining activity as compared to the control, sample kept at constant pH of 7.4, with 100% activity.

4.5 Isoelectric focusing of C-serum *Hevea* latex lectin binding protein

The IEF was carried out in this experiment for determination of the pI value of CS-HLLBP by using the desalted CS-HLLBP sample from the peak fraction of DEAE-Sephacel column, as described in the Method. An amount of 0.5 μg of CS-HLLBP was applied on the polyacrylamide gel isoelectric focusing. After the focusing was completed, the gel containing both standard protein markers and CS-HLLBP were removed from the supporting film, stained for 1 hour and followed by destaining until the background was at minimum in order to visulized the protein staining pattern of the IEF gel. The relative pI of the CS-HLLBP band was estimated from the standard protein markers band. The band position of CS-HLLBP is very close to that of typsin inhibition which served as standard pI marker of 4.7. Hence, the pI of CS-HLLBP was estimated to be about 4.7. The pI value of CS-HLLBP falls into the inhibitory pH range, between 4-5, observed under the pH stability study. Moreover, the pH range for optimum activity of CS-HLLBP, from 6-10, was well beyond its pI value.

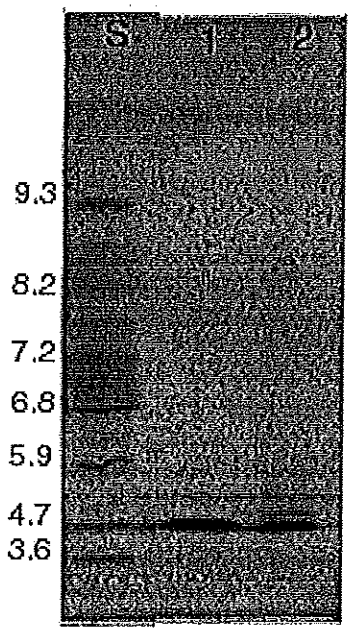


Fig. 32 pI determination of CS-HLLBP

S = standard marker pI 3.6-9.3

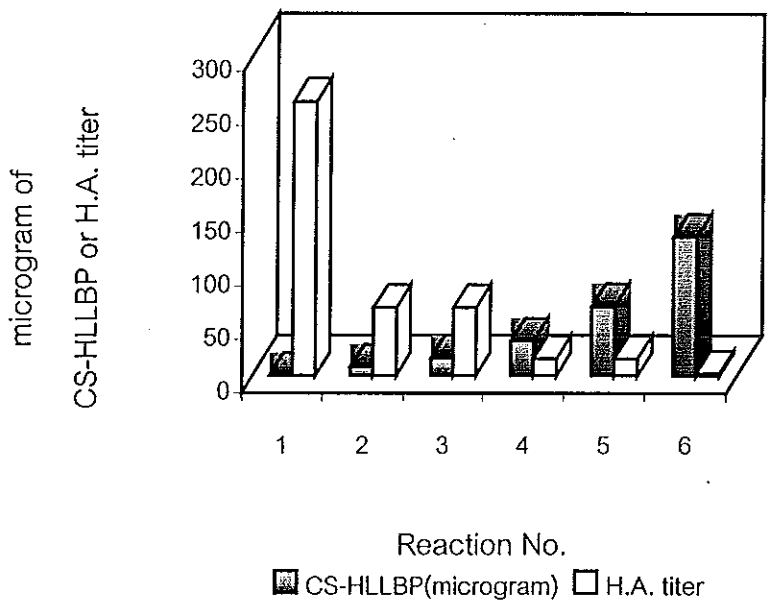
1 = standard marker pI 4.7

2 = purified CS-HLLBP

4.7 Hemagglutination inhibition specificity of C-serum *Hevea* latex lectin binding protein

The result Table 4 indicated that CS-HLLBP has the highest degree of specificity and recognition by HLL. In this experiment, the interaction between HLL and CS-HLLBP was tested by varying amounts of CS-HLLBP in the hemagglutination assay and monitored for the remaining hemagglutination acitivity of HLL.

Purified HLL containing 256 H.A. units of HLL was incubated at various amounts of serially diluted CS-HLLBP 8.13, 16.25, 32.5, 65, and 130 μg, in a total volume of 100 μl. After the incubation for 30 min the remaining H.A. activity of HLL was determined. The results in Fig. 33 indicate that the ability of CS-HLLBP in inhibiting hemagglutination activity of HLL was directly dependent on its concentration and being more pronounced at the initial phase or low concentrations.



Reaction number	1	2	3	4	5	6
μg CS-HLLBP	0	8.13	16.5	32.5	65	130
Remaining H.A. unit	256	64	64	16	16	2
% H.A. inhibition	100	75	75	93.75	93.75	99.3

Fig. 33 Inhibitory effects of various concentrations of CS-HLLBP on hemagglutination activity of HLL.

The 50 μl purified HLL containing 256 H.A. titer was incubated with 50 μl of various amount of partially purified CS-HLLBP as indicated for 30 min at room temperature. At the end of incubation, a portion of 50 μl of the mixture was taken for measuring the remaining activity of HLL. The control was done in the same way by using buffer instead of CS-HLLBP.

4.8 Effect of glycosidase treatments on C-serum *Hevea* latex lectin binding protein activity.

HLL was earlier shown to have specific binding with CS-HLLBP. The glycoconjugate portion of CS-HLLBP was assumed to be involved in the binding.

Several of glycosidases such as galactosidase, glucosidase, neuraminidase and chitinase were incubated with CS-HLLBP to remove or hydrolyze its carbohydrate portion, described under the Methods. The effect of glycosidase treatment was determined from the disappearance on hemagglutination inhibition activitiy of CS-HLLBP. An effective glycosidases will hydrolyze or remove certain portion of the glycoconjugate from the CS-HLLBP molecule, rendering it ineffective in binding to HLL. The effect of different glycosidases used for CS-HLLBP treatment was expressed as % remaining of H.I. activitiy as shown in Table 8.

It was found that chitinase was the only effective enzyme in causing CS-HLLBP to loose its binding capacity with HLL. Galactosidase and glucosidase were ineffective, even at higher amounts employed than that of chitinase. This suggests that neither galactose nor glucose is involved HLL-binding. Chitinase was very effective in decreasing CS-HLLBP binding capacity, 75% of its activity was abolished upon using only 0.125 units of the enzyme. Since chitin is very sensitive to chitinase,

this finding suggests a presence of similar carbohydrate moiety (N-acetylglucosamine) in the CS-HLLBP glycoconjugate.

Table 8 Effect of glycosidase treatments on hemagglutination inhibition activitiy of CS-HLLBP

Treatment	% Recovery on H.I. activity
1 = CS-HLLBP* (control)	100
1 = 1 + Glucosidase (5U)	100
2 = 1 + Galactosidase (50U)	100
3 = 1 + Chitinase (0.125U)	25

* The concentration of CS-HLLBP used for the incubation was 0.58 mg/ml in the total volume of 100 μl

4.9 Chitinase effect on C-serum *Hevea* latex lectin binding protein activity

In order to test for the degree of CS-HLLBP sensitivity to the chitinase hydrolysis, an experiment was carried out by treating CS-HLLBP with various concentrations of chitinase. The remaining activity of CS-HLLBP was expressed as % of remaining H.I. activity and shown in the Fig. 34. It was found that CS-HLLBP was very sensitive to chitinase, 50% activitiy dissapeared when only 0.005 unit of chitinase employed. This indicated that the carbohydrate moiety of CS-HLLBP was specifically sensitive and specific to this enzyme hydrolysis. Hence, N-acetylglucosamine might be part of the active carbohydrate components of the CS-HLLBP that required for HLL-binding recognition.

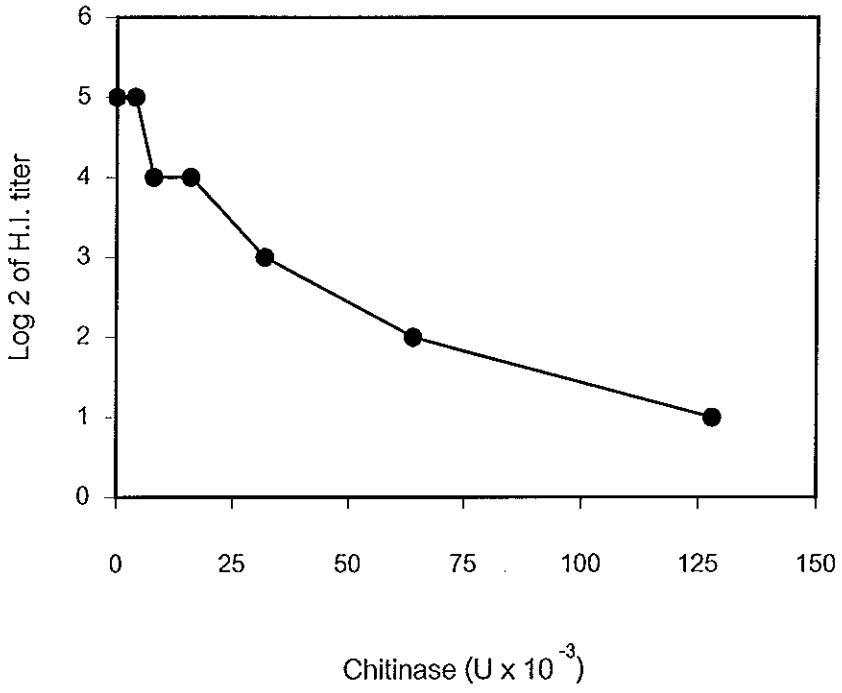


Fig. 34 Chitinase effect on hemagglutination inhibition of CS-HLLBP activity

Effect of chitinase on hemagglutination inhibition activity of CS-HLLBP was evaluated by incubating CS-HLLBP with various concentrations of chitinase as indicated. After the incubations period, the remaining hemagglutination inhibition activity was measured and compared with the control which was done under the same way by replacing chitinase by assay buffer.

4.10 Precipitin reaction between *Hevea* latex lectin and its binding proteins.

The specific binding between HLL and its binding protein from C-serum and small rubber particles was determined by using precipitin reaction. The purified HLL (50 μl) was mixed with (50 μl) of a several of 2-fold serially diluted solution of CS-HLLBP and SRP-HLLBP. The reactions were incubated at 37 °C for 4 hours then they were stored at 4 °C for 48 hours. The resulting pellet was collected, washed and subjected to SDS-PAGE. The protein bands was visualized after Coomassie Brilliant blue staining as shown in the Fig. 35 and 36.

Under an optimum condition for specific precipitation reaction protein with high affinity to HLL should be seen in the washed precipitate obtained. In the presence of C-serum, 40 kD protein band was predominately observed in the (washed) precipitate formed and the only band obseved in the most (serially) diluted sample (Fig. 35). This indicated strong binding affinity between the HLL and 40 kD protein which was earlier shown to be the CS-HLLBP.

Similarly, strong affinity between the HLL and small rubber particles proteins was predominately observed with 14 and 24 kD proteins of rubber particless. The 24 kD protein was reported as *Hevea* latex lectin binding protein from small rubber particle (Rukseree, 1998). When fixed

amount of HLL was used, the size of protein pellet obtained was directly proportional to the available amount of its binding proteins.

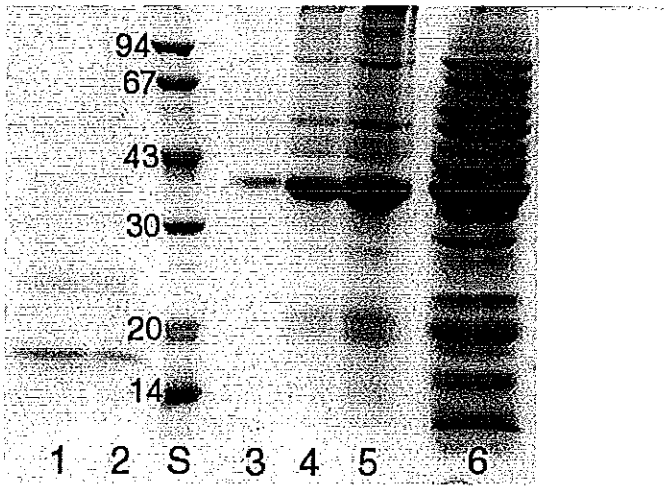


Fig. 35 SDS-PAGE of protein pellet obtained after the precipitin reaction

S = standard markers;

1,2 = purified HLL;

3-5 = serially diluted fraction washed protein pellet obtain after precipitin reaction;

6 = 60-80% $(NH_4)_2SO_4$ pellet fraction (C-serum).

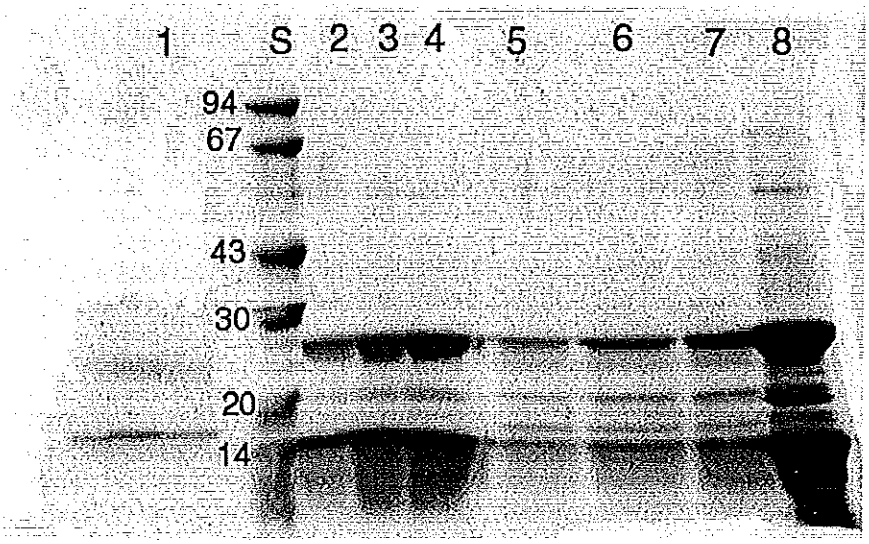


Fig. 36 SDS-PAGE of protein pellet obtained after the precipitin reaction.

S = standard markers;

1 = purified HLL;

2-4 = serially diluted fraction of washed protein pellet obtained after precipitin reaction;

5-6 = serially diluted fraction of remaining supernatant after the protein pellet removals.;

8 = proteins extracted from rubber zone 2.

Part 5. Rubber particles aggregation by *Hevea* latex lectin and its inhibition

The results from earlier part (1-4) revealed that HLL is a membrane bound lectin. It required a non-ionic detergent in the membrane extraction and column elution steps. Moreover, it was found to recognize glycoconjugates of latex proteins both soluble (CS-HLLBP) and non-soluble on rubber particles bound forms (14 and 24 kD).

In order to study the competitive bindings between these soluble and non-soluble HLL-binding proteins, a new quantitative method for assay of rubber particles aggregation was thus developed. A sample containing washed small rubber particles (SRP) was directly incubated with purified HLL. The resulting rubber particles aggregate formed was separated in a hematocrite centrifuge and visualized under a microscope as described under Method.

5.1 *Hevea* latex lectin in aggregation of small rubber particles by HLL

Small rubber particles aggregation was clearly seen in assay mixture containing fixed amount of SRP and HLL solution. Only basic Fuchsin, but not Alcian blue, was found to be successful in the staining of rubber particles aggregates. No stained rubber aggregate was observed in the control assay where lectin was omitted (Fig. 37). The amount of rubber aggregates formed is direclty proportional to the incubation time (Fig. 38). Other non-lectin related proteins such as bovine serum albumin, latex C-serum protein and fetuin, failed to induce the rubber particles aggregation (Fig. 39)

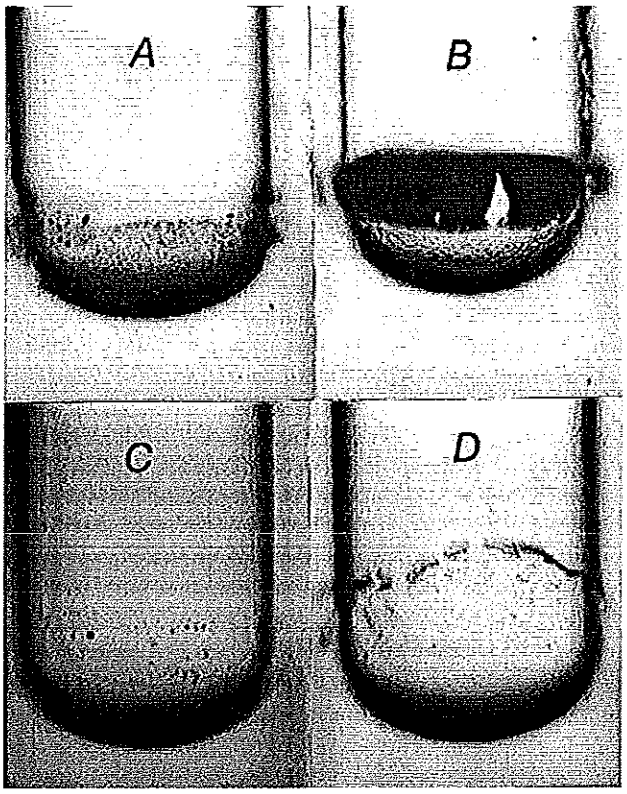


Fig. 37 Effect of HLL on rubber particle aggregation. After 30 min of incubation, the SRP mixture was stained with either basic Fuchsin (A&B) or Alcian blue 8GX (C&D), SRP aggregate was visualized under a microscope. The OD_{600nm} of SRP solution employed in the assay was between 0.8-0.9.

A, C = SRP + TBS = control

B, D = SRP + 1.8 μg HLL

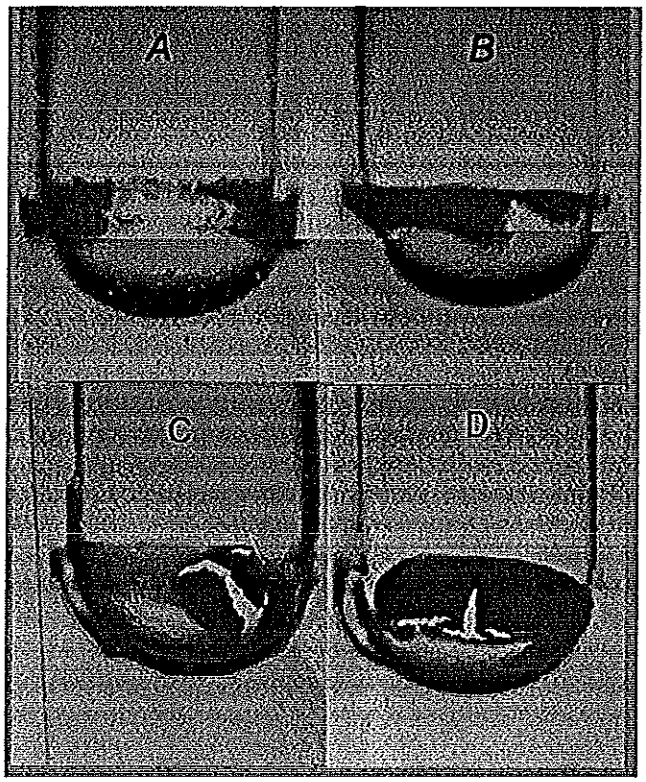


Fig. 38 Time course on SRP aggregate formation as induced by HLL. The incubation mixture containing SRP and HLL (1.8 μg) was incubated for 0, 10, 20, 40 min as in A, B, C and D, respectively. The OD_{600nm} of SRP solution was between 0.8-0.9 and basic Fuchsin was used for staining.

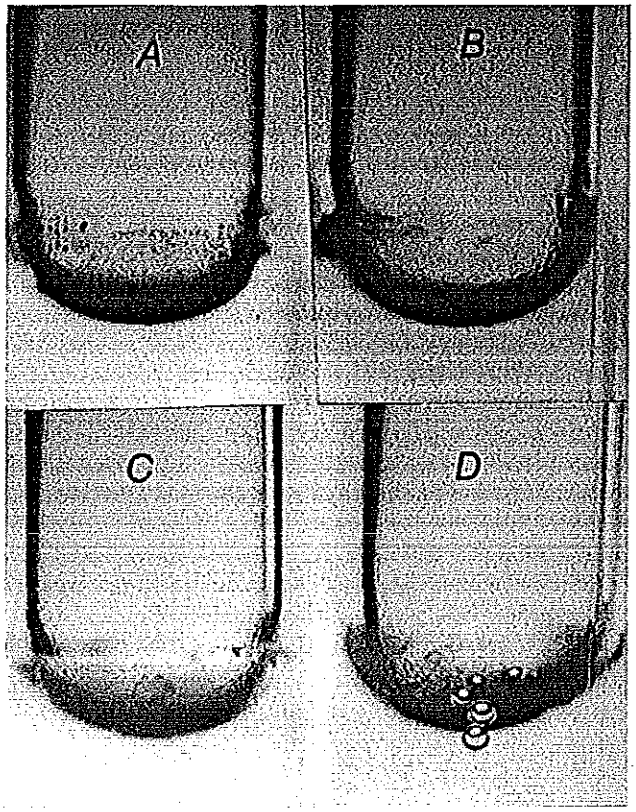


Fig. 39 Effect of various proteins other than HLL on rubber particles aggregation. The OD_{600nm} of SRP solution was between 0.6-0.8. The total volume of incubation mixture was 50 μl.

A = SRP + TBS

B = SRP + BSA (10 μg)

C = SRP + fetuin (2.5 μg)

D = SRP + C-serum protein (8 μg)

5.2 Inhibitory effect of C-serum *Hevea* latex lectin binding protein on degree of rubber particles aggregation induced by *Hevea* latex lectin

From the results in part 4.6, the CS-HLLBP was shown to have very strong inhibition on hemagglutination induced by HLL. In this experiment, the effect of interaction between HLL with varying amount of CS-HLLBP was verified from the inhibitory degree on rubber particles aggregate formation.

The degree of HLL induced SRP aggregate formation was reduced with increasing amount of added CS-HLLBP (Fig. 40). This indicated that CS-HLLBP was more effective in HLL-binding than that of SRP-HLLBP. This CS-HLLBP may play an important role in preventing rubber particles aggregation and hence promoting latex flow upon tapping.

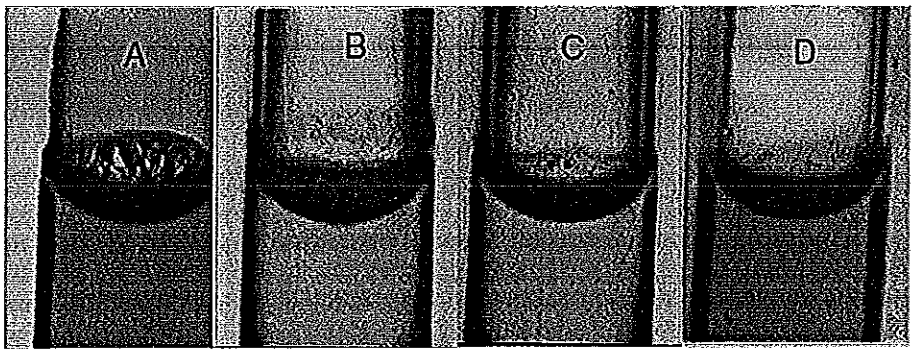


Fig. 40 Inhibitory effect of CS-HLLBP on degree of HLL induced rubber particles aggregate formation. The OD_{600nm} of SRP solution was between 0.6-0.8.

A = (SRP + TBS) + HLL 5 μg

B = (SRP + CS-HLLBP, 16.5 μg) + HLL 5 μg

C = (SRP + CS-HLLBP, 65 μg) + HLL 5 μg

D = (SRP + CS-HLLBP, 130 μg) + HLL 5 μg

5.3 Rubber particles aggregation by commercial lectins

Earlier findings from H.I. study showed that HLL has specific binding to sugar residues conjugated to glycoprotein from various sources such as (asialo) fetuin and (asialo) mucin as well as amino sugar polymer of chitin. In this experiment various commercial lectins with different sugar binding specificities were tested for their abilities in aggregating rubber particles (Table 9). All lectins were practically unable to agglutinate SRP except for *Erythrina cistagalli*, recognizing sugar dimer of β-gal(1→ 4) glcNAc, showed slight ability to agglutinate SRP as compared to HLL.

Table 9 Effect of commercial lectins in inducing small rubber particles aggregation as compared to HLL

Lectin	Specific binding	SRP-aggregation
Hevea latex lectin (HLL)	(see Table 4)	+4 (100%)
Concanavalin A	α-D-glucosyl	0 (0%)
Erythrina cristagalli	β-gal(1→ 4) glcNAc	+1 (25%)
Maackia amurensis	sialic acid	0 (0%)
Sambucus nigra	NeuNAc2→6 gal/galNAc	0 (0%)
Cicer arietinum	Fetuin	0 (0%)

The concentration used was 1 mg/ml except for HLL, 0.58 mg/ml

5.4 Correlation study between activity levels C-serum *Hevea* latex lectin binding protein and rubber yield per tapping

From our previous results, HLL showed very strong Binding affinity to CS-HLLBP. It, however, failed to induce rubber particles aggregation if CS-HLLBP was highly present. This result suggests an *in vivo* function of CS-HLLBP in preventing rubber particles aggregate or plug formation. Therefore, it is interesting to study the correlation between CS-HLLBP and rubber yield per tapping. In this experiment, latex samples were collected from nine rubber trees giving high, medium and low levels of rubber yield. After ultracentrifugation of fresh latex, the C-serum was separately prepared and used to quantitate for its respective hemaggultination inhibition activity. The result as shown in Fig. 41, revealed a high correlation value ($r = 0.97$) between the total hemagglutination inhibition activity of CS-HLLBP and rubber yield per tapping. This result supports an important role of CS-HLLBP in prolonging latex vessel plug formation to impede its flow upon tapping.

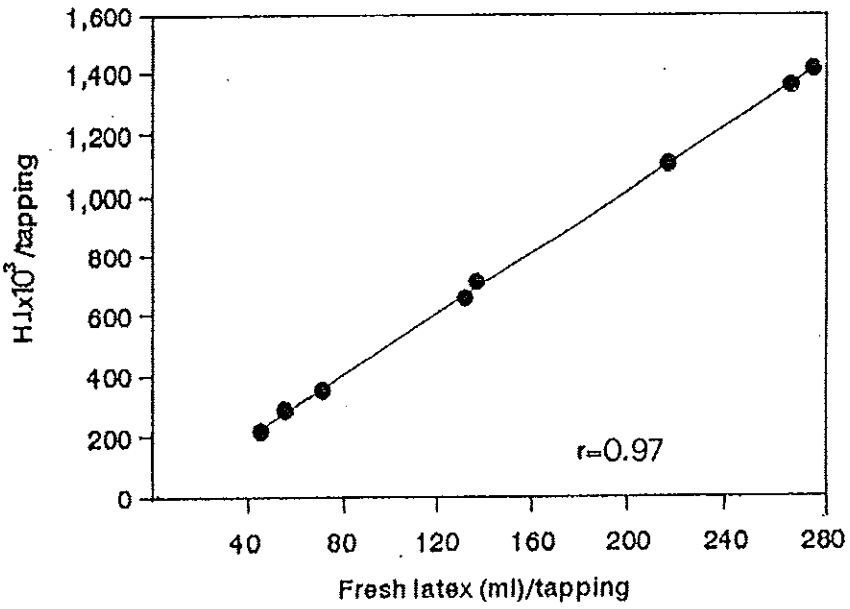


Fig. 41 Correlation between levels of CS-HLLBP and rubber yield per tapping

Latex C-serum prepared from nine rubber trees giving different volumes of latex yield per tapping as indicated. The H.I. levels were determined from respective C-serum samples. The correlation value between the total hemagglutination inhibition activity of CS-HLLBP and rubber yield per tapping was calculated to be 0.97

Chapter 4

Discussion

After tapping, latex flows out from the severed latex vessels of the phloem tissue over a period of time. Most exuded latex are delocalized from vessels near the cut. The rubber tree has ability to limit its loss of material after wounding by inducing flow stoppage. This characteristic varies from tree to tree as well as clone to clone. The tree with highly efficient mechanisms of wound response give off less latex yield. Many studies had been attempted to reveal the mechanism involved in latex vessel plugging leading to flow stoppage. Southorn had shown from his microscopic examination of the tapping region following flow stoppage that the vessels were covered by a thick cap of rubber coagulum consisting of many damaged lutoids (Southorn, 1968). This suggested that both breakage lutoid and rubber particles are required for forming physical plug of rubber coagulum. The mechanism of aggregation or coagulation formed between rubber particles and lutoid membrane debris is presently not well understood or still unclear. We proposed to see whether a certain membrane bound lectin is involved in this type of coagulation process. In our study, we have found that lutoid membrane possessed a bound lectin which was extractable by non-ionic detergent (Triton X-100). It is

shown to be able to agglutinate rabbit erytrocytes and its hemagglutination could be inhibited by various glycoproteins. It was thus clear that the lutoid membrane contained lectin or so called HLL, with specific recognition and interaction with erythrocyte glycoproteins. When rubber particles was tested with HLL in a similar manner as hemagglutination assay, it was found that this lectin could strongly agglutinate the rubber particles. This is very interesting and important *in vitro* observation, which supports the hypothesis that coagulum or plugs formation involved lectin and glycoprotein interaction. Moreover, we have found that the activity of HLL was inhibited by a glycoprotein purified from the C-serum fraction of ultracentrifuged fresh latex. This glycoprotein namely CS-HLLBP is different from another *Hevea* latex lectin binding protein found on the small rubber particles (SRP-HLLBP) (Rukseree, 1998). Under the same assay condition, the CS-HLLBP had a much higher specific H.I titer than SRP-HLLBP. This indicated that the affinity binding between HLL and its cytosolic glycoprotein is difficult to replace or complete by another glycoprotein counterpart located on rubber particles.

Part 1. Preparation and purification of *Hevea* latex lectin from lutoid membrane

The methods employed for the purification of lutoid membrane proteins are modified from those generally used to purify water-soluble, nonmembrane-associated proteins. These methods include precipitation, gel filtration , ion-exchange, reversed-phase and affinity chromatography.

The non-ionic detergent (Triton X-100) was needed in extraction of HLL. This suggest that it is tightly bound to lutoid membrane by strong hydrophobic interaction. Since most of the non-ionic detergents generally have low critical micelle concentration (cmc) (< 1 mM) and are difficult to remove without using special resins such as Bio-Rad SM-2 beads. We therefore, incubate the extract with SM2 to remove residual Triton X-100 before further purification on ion-exchange chromatography. Triton X-100 was revealed to be necessary for HLL elution. Although its absorbance at 280 nm had drastic interference effect on the wavelength used to monitor the chromatographic elution profile of the protein but at the minimum detergent concentration used, it had only little interference on the hemagglutination assay employed to monitor HLL activity.

In general, integral membrane proteins are predominantly amphiphiles with hydrophilic regions exposed to the aqueous enviroment and hydrophobic regions embedded in the lipid matrix. The phase separation in the presence of detergent under Method B indicated that the HLL was a membrane-

bound lectin. Under suitable condition on inducing aqueous two phase system between detergent and salt, the mebrane lectin was found in the detergent phase. Moreover, the Triton X-100 extract from membrane-detergent phase revealed a distinct protein pattern consisting of two major proteins of approximately 30 kD and 17 kD, as analyzed by SDS-PAGE (Fig .20). These are the two main proteins that bound to lutoid membrane. Moreover, It was found that the repeated washing of lutoid membrane was needed to remove the contaminated soluble proteins. If lutoid membrane was not washed by TBS before extraction by Triton X-100, the unwashed protein contained many contaminated and unrelated proteins. These proteins are from C-serum, B-serum, Frey-wyssling and including minor proteins on rubber particless (Yeang *et al.*, 1996).

The purification protocol for HLL summarized in Table 2 and 3 indicated that the Method B is more suitable for handing large-scale preparation of partially purified HLL with slighly lower purification fold and specific activity than that obtained under Method A.

Part 2. Characterization of *Hevea* latex lectin

2.1 Molecular weight determination

The determination of the overall state of association of a protein requires the measurement of its native molecular weight. Gel

permeation chromatography is a convenient method to characterize the size of a detergent protein complex.

In the study on characterization of HLL, its native molecular weight was determined by gel filtration in the presence of Triton X-100, and calibrated to be 412 kD. Its subunit molecular weight was determined to be 17 kD on SDS-PAGE. The results suggested that the native HLL of 412 kD contains about 24 subunits of 17 kD. This multimeric nature observed with HLL was similar to another membrane bound lectin that had been purified from human liver by Baenziger and Maynard (1980). They found association of subunit with M_r 41,000 into a complex of large molecular weight when examined by gel filtration in the presence of Triton X-100.

This is unlike another latex lectin purified from *Euphorbia neriifolia,* which appeared to be a dimer with M_r 60 kD on gel filtration and showing a single band at M_r 32 kD in SDS-PAGE (Seshagirirao and Prasad, 1995).

2.2 Hemagglutination property of *Hevea* latex lectin

The HLL could agglutinate erythrocytes from rabbit and mice but not human. This erythrocyte specificity is in accordance with property of a lectin purified from the latex of *Euphorbia marginata* (Stirpe *et al.*, 1993) and another two lectins (HOL-I and HOL-II) isolated from the marine sponge *Halichondria okadai*, (Kawagishi, 1994). Similary, a lectin purified from the tuber of *Arum maculatum* (family Araceae) was

shown to readily agglutinate rabbit erythrocytes but had no effect on human erythrocytes even when a 100-times greater amount of lectin was applied (Allen, 1995). Moreover, Sandhu *et al.* (1990) had showed that the lectin extracts of eleven members of the same (Araceae) family were all capable of aggutinating rabbit, guinea pig, rat and sheep erythrocytes but not human erythrocytes. They suggested that the human red cells might have few terminal N-acetyllactosamine groups on their glyco-proteins.

The hemagglutination activity of HLL was heat stable upto 60 °C. A sharp decrease in its acitivity was observed when temperature raised from 60 °C to 80 °C similar to *Hevea* bark lectin (Wititsuwannakul, 1998). This heat stable property is quite similar to lectin from elder bark (*Sambucus nigra* L.) with M_r of 140 kD that was heat-stable upto 55 °C and completely inactivated at 70 °C (Brokert *et al.*, 1984). The HLL-induced hemagglutination was not markedly affected by variation of the pH and its maximum activity was retained over the broad pH range 5-10. Moreover, the protease or alkaline (0.5 N NaOH) was not able to destroy its native activity. In general, the proteins with stability over a broad pH range are usually resistant to wide range of poteolytic enzymes and most of them are also fairly heat-stable. These properties of HLL are similar to many other plant lectins that are known to exhibit similar resistance against unfavourable conditions and a majority of plant lectins

are not degraded by animal or insect gut proteases. For example, the lectin from stinging nettle (*Urtica dioica*) rhizomes is stable in extreme pHs, 5% (w/w) trichloroacetic acid and in 0.1 N NaOH. It can withstand boiling water and is reported to be resistant in its native form against all commonly used proteases (Peumans *et al.*, 1984 a).

The carbohydrate binding specificity of HLL was determined with a series of simple sugars, their derivatives and glycoprotein (Table 4-5). From these results, HLL can be classified as a sialic acid binding lectin since the glycoproteins containing sialic acid (fetuin and mucin) had more specific binding to HLL than glycoproteins without sialic acid (asialofetuin and asialomucin). Moreover, this was supported by the result of treated BSM with neuraminidase that the inhibitiory activity of BSM was abolished by 50 % after incubation with neuraminidase for 5 min at 37 °C.

2.3 Rubber particles agglutination property of *Hevea* latex lectin

The rubber aggregation induced by HLL was clearly shown in this study. The aggregated rubber particles were intensely stained by basic Fchsin but not Alcian blue dye generally employed for detection of glycoprotein. The amount of rubber aggregates formed was directly proportional to the incubation time. Other non-lectin related proteins such as bovine serum albumin, latex C-serum protein and fetuin, failed to induce the rubber particles aggregation. The result indicated that HLL specifically

by bind to the carbohydrate protein of glycoprotein located on rubber particles in causing latex coagulation.

Our finding indicated that a real lectin (HLL) is functioning in inducing rubber particles aggregation. This is different from earlier report where lectin-like protein, hevein, was suggested to be involved (Gidrol *et al.*, 1994). The amount of HLL-induced rubber aggregate formation was reduced with increasing amount of added CS-HLLBP. The HLL binding protein isolated from C-serum, called CS-HLLBP, was shown to be a glycoprotein with molecular weight of 40 kD. This glycoprotein was extensively characterized for its various properties as shown in results of the preceeding parts. It was also found that this glycoprotein had strong inhibition on hemagglutination activity of HLL.

Part 3. The purification and characterization of C-serum *Hevea* latex lectin binding protein

Fresh natural latex from *Hevea brasiliensis* contains about 0.95% proteins of which 27.2% is in the rubber fraction, 47.5% in the serum fraction and 25.3% in the bottom fraction (Tata, 1980) Several reported C-serum proteins are enzymes that could be classified under oxidoreductase, transferase, hydrolase, lyases and isomerase (Jacob and Prevot, 1989). However, the most abundantly found protein reported in C-serum was a non-enzymic protein known as α-globulin (Archer, 1955). It was an acidic

protein, pI of 4.55 and easily absorbed at the surface of rubber particles (Archer, 1955). Recently, another acidic protein (pI = 3.5) in C-serum fraction of the latex with an apparent molecular mass of 25 kD on SDS-PAGE was found by IgE immunoblotting to be reactive in 52% of latex-allergic patient sera (Akasawa *et al.*, 1995).

In this study, we have isolated a glycoprotein form C-serum namely CS-HLLBP that could inhibit heamaggutination activity of HLL. This protein was observed to be most predominant C-serum protein obtained under ammonium sulfate fractionation (Fig. 27) and quantitatively similar to α-globulin, earlier reported as most abundant C-serum protein. The result on MW analysis indicated that CS-HLLBP is a large homomultimeric protein composing of 6 subunits of 40 kD.

The thermal stability study indicated that CS-HLLBP was quite heat-stable and only half of its activity lost at 70-80 °C. This supports the generally known concept that glycoprotein is normally more thermostable than its simple (unmodified) protein form. The optimum pH stability of native CS-HLLBP was between 5-8 and the protein retained only half of its activity at pH 4. The pI value was found to be 4.7 and very close to the value reported for α-globulin, a major C-serum protein (Archer, 1955).

The importance of carbohydrate moiety of CS-HLLBP was evaluated from the effect of several glycosidase treatments. It was found that only chitinase was effective in causing CS-HLLBP to loose its activity while

other glycosidases (galactosidase, glucosidase and neuraminidase) were ineffective. This suggested that the CS-HLLBP is a glycoprotein and carbohydrate moiety of CS-HLLBP is somewhat similar to chitin and thus sensitive to hydrolysis by chitinase. The exact carbohydrate structure of this glycoprotein was not known and beyond the scope of this work. However, the result suggested that N-acetylglucosamine might be the active component of carbohydrate part of this glycoprotein.

The general properties of the *Hevea* latex lectin binding protein purified from C-serum and small rubber particles localized in zone 2 rubber layer of centrifuged latex were somewhat different. The purified SRP-HLLBP was reported to possess native and subunit molecular weight of 120 kD and 24.5 kD upon native PAGE and SDS-PAGE analyzes, respectively. The pI value was determined to be 5.2 while pH stability was in the range of 5-8. It was a thermostable protein and could stand heat treatment upto 60 °C (Rukseree, 1998). Whereas, the values of native and subunit MWs of CS-HLLBP were found to be higher than those reported for SRP-HLLBP. Similar to SRP-HLLBP, the CS-HLLBP is also an acidic protein but having a higher pI value of 4.7. Under the same condition, the specific H.I. titer of CS-HLLBP was ten times higher than that of SRP-HLLBP.

In addition, CS-HLLBP showed a dose-dependent mannner on inhibition of HLL induced agglutinations of both rabbit erythrocytes and rubber particles. This indicated that HLL had a stronger affinity binding for

the carbohydrate proteins localized on CS-HLLBP than those on either rabbit erythrocytes or rubber particles. This suggested that CS-HLLBP was the natural soluble intrinsic binding protein for HLL and might represent its specific function as an anticoagulating factor. Accordingly, a highly positive correlation was found between activity levels CS-HLLBP and rubber yield per tapping ($r = 0.97$) indicating its important role in prolonging latex vessels plug formation for impeding latex flow upon tapping. In another word CS-HLLBP is a latex flow promoter.

Part 4. A proposed model for rubber particles aggregation.

Our finding showed that HLL is a hydrophobic lectin which bound to lutoid membrane. The exposure of its binding site may follow after lutoid membrane rupture. Rubber particles are introduced into coagulum formation by lutoid membrane through specific interaction between HLL located on lutoid membrane and its binding glycoprotein located on rubber particles. These associations are, however, inhibited by the soluble HLL binding protein (CS-HLLBP), the most abundant protein found in the soluble C-serum. Moreover, a high correlation between levels of CS-HLLBP and rubber yield per tapping was observed. This indicates an important role of CS-HLLBP in prolonging latex vessel plug formation in impeding the latex

flow upon tapping. From these results, a model for coagulation of latex is, therefore, proposed and depicted under Fig. 42.

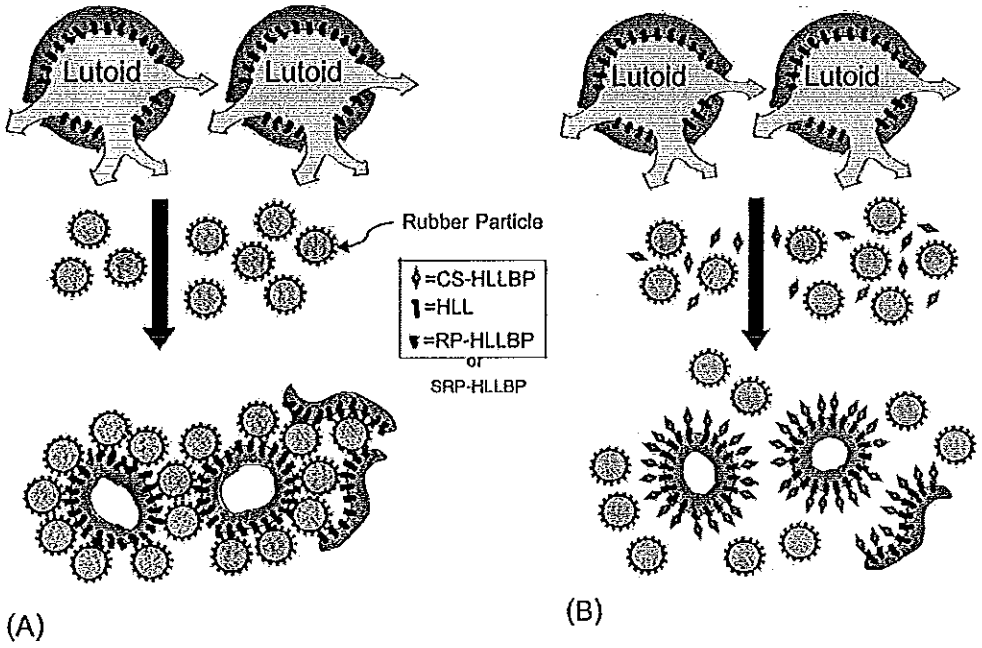


Fig. 42. Model for coagulation of rubber latex

Latex coagulation takes place at the wounded sites distal open-end of latex vessels at the tapping cut. The bursting of lutoid particles lead to the exposure of lutoid membrane bound lectin (HLL) which can bind to either its 24 kD-glycoprotein receptor (SRP-HLLBP) on rubber particles, forming a coagulum as depicted in process A or the soluble 40 kD-glycoprotein (CS-HLLBP) in the latex cytosol, forming no coagulum as depicted in process B. The latex coagulation takes place whenever process A overcome process B i.e. at the tapping site.

Although many carbohydrate-binding proteins are not widely uses as biochemical tools in a growing member of applications, little is known about their physiological function in plant. Our observation reveals significant physiological function in *Hevea* latex lectin as a RP aggregation or coagulating factor (Fig. 43) and in additions, the most abundant globulin protein as an anti-RP aggregating or anti-coagulating factor (Fig. 44).

Our present proposed model is different from the one earlier proposed by Gidrol *et al.*, 1994. According to their model, hevein, a soluble protein inside lutoid particles, acts as a lectin-like protein in bringing together rubber particles. Both hevein and chitinase, found within lutoid were suggested to play opposite function, hevein served as coagulating factor while chitinase as anti-coagulating factor, respectively. In contrast to their model which excludes the presence of damage lutoid, our model demonstrates its involvement in coagulum formation.

Moreover, our model not only showed that the coagulating factor (HLL) and anti-coagulating factor (CS-HLLBP) are localized in different compartments but we also found that the chitinase showed both positive and negative influences on the latex destabilization by inhibition SRP-HLLBP on rubber particles (Rukseree, 1998) and CS-HLLBP in C-serum from HLL binding, respectively.

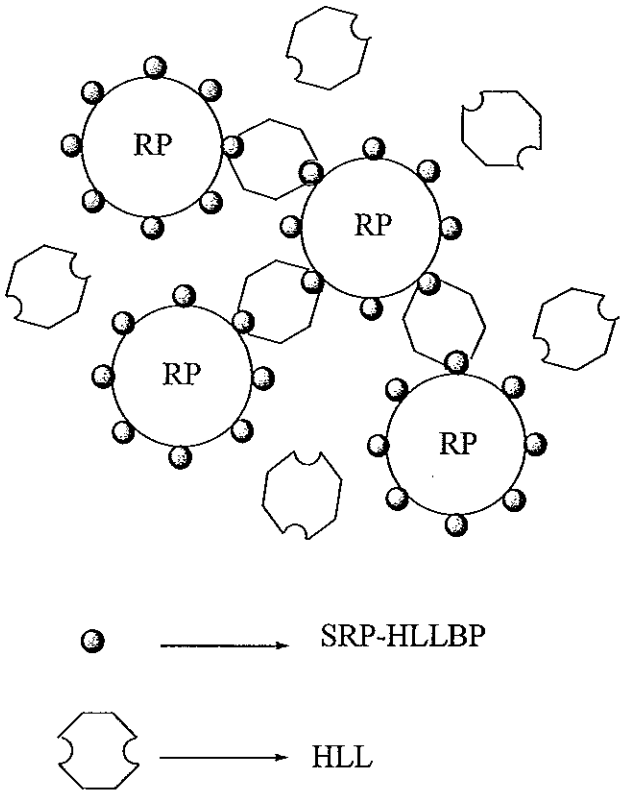


Fig. 43. HLL as a RP-aggregating or coagulating factor

The HLL isolated from lutoid membrane was shown to have specific binding with proteins located on surface of small rubber particles (SRP-HLLBP).

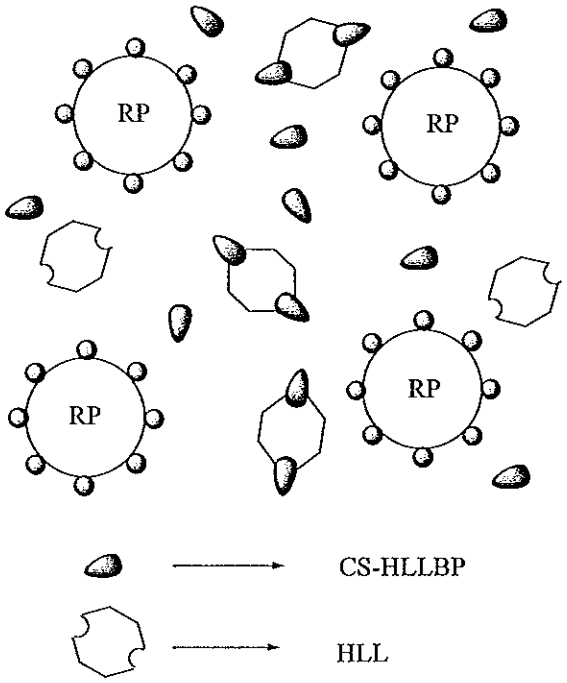


Fig. 44. CS-HLLBP as an anti-RP-aggregating or anti-coagulating factor.

A soluble 40 kD-glycoprotein (CS-HLLBP) in the latex cytosol was shown a specific binding with HLL.

Chapter 5

Summary

The result of this investigation could be summarized as folllow :

1. *Hevea* latex lectin (HLL) was extracted and homogeneously purified from lutoid membrane obtained from bottom fraction of ultracentrifuged fresh latex.
2. A non-ionic detergent was required for HLL extraction as well as elution from column chromatography.
3. Purified HLL possesses native and subunit M_r of 412 kD and 17 kD upon gel filtration chromatography and SDS-PAGE analyses, respectively.
4. HLL showed higher specific binding with glycoproteins containing sialic acid than glycoprotein without sialic acid.
5. HLL was a thermostable protein and stable upto 60 °C upon heat treatment without any activity lost.
6. The pH stability of HLL was in the broad range from 5-8.
7. HLL was stable to proteolytic (pronase) and alkaline (0.5 N NaOH) hydrolysis.
8. HLL could agglutinate both rabbit erythrocytes and rubber particles.

9. The activity of HLL was inhibited by CS-HLLBP, a glycoprotein abundantly found in C-serum fraction of ultracentrifuged fresh latex.
10. CS-HLLBP was able to inhibit rabbit erythrocytes and rubber particles aggregation induced by HLL.
11. CS-HLLBP possesses native and subunit M_r of 240 kD and 40 kD upon gel filtration chromatography and SDS-PAGE analyses, respectively.
12. The pI value of CS-HLLBP, as determined by isoelectric focusing (IEF) was 4.7.
13. The pH stability of CS-HLLBP ranged from 5-8.
14. CS-HLLBP was sensitive to chitinase hydrolysis, resulting in complete activity lost.
15. The correlation value obtained between activity levels CS-HLLBP and rubber yield per tapping is very high ($r = 0.97$).
16. HLL of lutoid membrane is suggested to function as rubber latex coagulating factor.
17. CS-HLLBP of cytosolic latex protein is suggested to function as an anti-coagulating factor.

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