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A role for a *Hevea* latex lectin-like protein in mediating rubber particle aggregation and latex coagulation ☆

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Abstract

An *in vitro* aggregation of washed lutoid membrane and rubber particles, respectively, prepared from the bottom (lutoid) fraction and rubber layer of centrifuged fresh latex, leading to the formation of rubber coagulum necessary for a latex coagulation was demonstrated. A Triton X-100 extract of washed lutoid membrane proteins, isolated and prepared from the bottom fraction of centrifuged fresh latex was examined for its role in the latex coagulation process. It induced agglutination of rabbit erythrocytes, indicating the presence of a lectin-like protein. *Hevea* latex lectin-like protein (HLL) was purified to homogeneity by active chitin binding separation, followed by DEAE-Sepharose chromatography. Its M_r analyzed by SDS–PAGE was 17 kDa, whereas that determined by gel filtration was 267 kDa. The HLL had a pI value of 7.2. Several glycoproteins were shown to inhibit the HLL-induced hemagglutination. The hemagglutinin activity of HLL was enhanced by Ca^{2+}. Of most interest was the finding that HLL strongly induced aggregation of the *Hevea* latex rubber particles (RP). This strong RP aggregation leads to latex coagulation, indicating the possibility that it is involved in the formation of the coagulum that plugs the latex vessel ends and stops the flow of latex upon tapping. In addition, the purified HLL also induced aggregation of RP taken from several other non-*Hevea* latex producing plants. This might indicate either a common or universal role of this lectin-like protein in RP aggregation and hence latex coagulation. This paper, for the first time, provides clear and unequivocal evidence for either a key biological role or physiological function of an endogeneous latex lectin-like protein in the sequential process of latex coagulation.

Keywords: *Hevea brasiliensis*; Euphorbiaceae; Rubber latex; Lectin; Agglutinin; Rubber particle; Latex coagulation

1. Introduction

Protein–carbohydrate interactions play a major role in establishing the specificities of a wide range of biological recognition and communication events. A class of carbohydrate binding proteins, called lectins, have been isolated from a wide variety of natural sources including plants, fungi, bacteria, viruses, algae and body fluid, as well as cell membranes of vertebrates (Goldstein and Hayes, 1978; Goldstein and Poretz, 1986; Lis and Sharon, 1986). Lectins in animal systems have been implicated as a direct first-line defence against pathogens, cell trafficking, immune regulation and prevention of autoimmunity (Hauri et al., 2000; Kilpatrick, 2002). The biological function of plant lectins is, nevertheless, still unclear although diverse roles that include enzymatic activity, storage proteins, cell wall

Abbreviations: RP, rubber particle; HLL, *Hevea* latex lectin-like protein; HBL, *Hevea* bark lectin; HA, hemagglutination activity.

☆ Part 1 in the series 'Proteins specifically involved in *Hevea* rubber particle aggregation and latex coagulation'.

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extension, transport of carbohydrate, mitogenic stimulation, as well as plant defense mechanism have been suggested (Etzler, 1985; Chrispeels and Raikhel, 1991; Van Damme et al., 1998). There has been almost no evidence to indicate the occurrence of plant lectins that recognize and bind 'endogenous' glycoconjugates or ligands, and are therefore probably involved in recognition mechanisms within the organism itself.

The rubber tree (*Hevea brasiliensis*) is one of the world's most frequently wounded plants. The tree is regularly tapped by means of bark stripping, for collecting rubber latex, throughout its economic lifespan. Freshly tapped latex is in fact a living cytoplasm in which the rubber particles and non-rubber particles such as lutoids and Frey–Wyssling, particles as well as other cell components are dispersed in an aqueous phase of the cytosol (Moir, 1959; Dickenson, 1969). The rubber latex is largely formed and stored in the rings of anastomosing lacticifers in the inner bark area. This allows draining of the latex from a large area after tapping (Zhao, 1987). A process leading to rubber latex coagulation at the severed vessel ends was suggested to take place immediately after tapping (Boatman, 1966). The progressive plugging of latex vessels during the course of the latex flow, following tapping, restricts the quantity of latex exuded and is thus an important limiting factor of yield output per tapping (Milford et al., 1969). The plugging of latex vessels is vital for the rubber tree in preventing the loss of its metabolites and entry of pathogens to the phloem. Various hypotheses have been proposed to explain the mechanism of plugging, many of which have emphasized a role for the damaged vacuolar lutoids (Southorn, 1969; Southorn and Edwin, 1968). Both damaged lutoids and rubber particles were found to be associated in a cap of rubber coagulum formed over the severed vessels (Southorn, 1968). A similar *in vitro* aggregation of ruptured lutoids and rubber particles that resulted in the formation of a rubber coagulum had also been demonstrated in ultracentrifuged fresh field-latex upon prolonged storage (Wititsuwannakul et al., 2004). A soluble protein inside the lutoid bodies, known as hevein, has also been shown to mediate rubber particle aggregation in a lectin like manner (Gidrol et al., 1994). This type of aggregation is not directly related to the association observed between lutoid membrane debris and rubber particles. It is the intention of this paper to investigate the presence of a latex protein on the lutoid membrane that could act like a lectin and induce aggregation of rubber particles to form a rubber latex coagulum.

2. Results and discussion

In this report, we demonstrate an *in vitro* aggregation of washed lutoid membrane and rubber particles giving rise to a rubber coagulum in a latex coagulation. Biochemical studies on a *Hevea* latex lectin-like protein derived from the lutoid membrane isolated from the bottom fraction of the centrifuged fresh latex are described. The role and function of the intrisic latex lectin-like protein and its involvement in the latex coagulation process was established.

2.1. In vitro aggregation of lutoid membrane and rubber particles

The bottom (lutoid) particles in a centrifuged fresh latex, comprising around 20% or more of the latex volume, had previously been shown to be either unstable or disappeared upon prolonged storage of the fresh latex. The membrane debris from disrupted bottom particles was shown to be associated with rubber particles as a puffy upper rubber phase in the destabilized latex (Wititsuwannakul et al., 2004). Washed lutoid membranes and rubber particles, prepared from centrifuged fresh latex, were also observed to undergo an *in vitro* aggregation (Fig. 1). After incubating washed lutoid membranes with a rubber particle suspensions for 30 min, the membranes

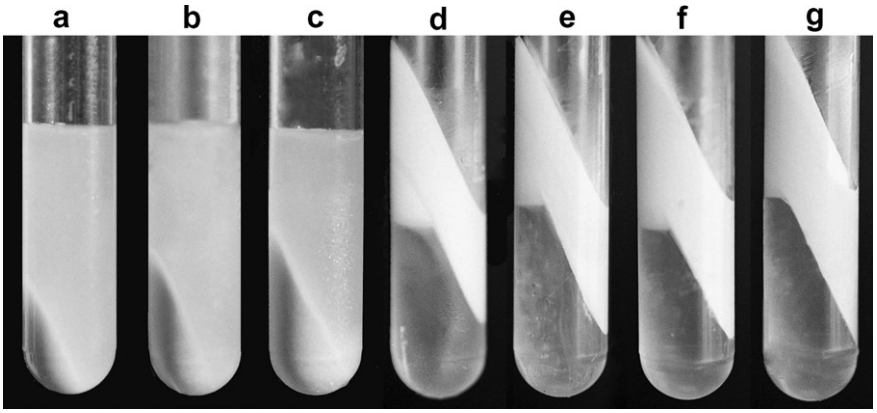

Fig. 1. Ultracentrifugation of suspensions, 6 ml each, containing washed lutoid membrane (a–c), washed rubber particles (d) and their mixtures (e–g). Tubes a, b and c: control washed lutoid membrane suspensions with total protein contents of 24, 48 and 72 mg and dry weight contents of 0.13, 0.26 and 0.39 g, respectively; Tube D: control rubber particle suspension with total protein content of 1.28 g and dry weight content of 2.42 g. Tubes e, f and g: a + d, b + d and c + d mixtures, respectively.

had completely disappeared from the bottom fraction and were found to be associated with the top rubber layer upon centrifugation. The increase in the size of the rubber layer was directly proportional to the amount of the washed lutoid membrane debris added to the incubation mixtures. (Fig. 1, tubes e–g). A direct involvement of lutoid membranes in rubber coagulum formation and latex coagulation was thus demonstrated. Although the lutoid membranes have always been implicated in the latex coagulation process, until now, with these findings, their role has neither been known nor well understood (Southorn, 1968, 1969a; Southorn and Edwin, 1968). Our current working hypothesis is that the latex coagulation process is based on a biologically specific recognition and interaction. We have therefore isolated a *Hevea* latex lectin-like protein from the lutoid membrane and investigated its properties.

2.2. Purification of Hevea latex lectin-like protein (HLL)

The HLL was extracted with Triton X-100 from the membranes of a washed lutoid particle sediment in the bottom fraction of the centrifuged fresh latex for purification and characterization of its properties. A Triton X-100 extract of the washed lutoid membrane proteins agglutinated erythrocytes from rabbits and mice, but had no activity with human erythrocytes of any blood group. The erythrocyte specificity of the lectin-like protein found in *Hevea* latex was in accordance with the property reported for the lectin from the latex of *Euphorbia marginata* (Stripe et al., 1993). Triton-X (0.2%) was as effective in solubilizing HLL from the vacuolar lutoid membrane as was the detergent previously employed for extracting lectin associated with the vacuolar membrane (Heyen et al., 2002). Triton-X was removed from the membrane protein extract using SM-2 beads prior to any further purification steps. HLL was found to have a high affinity for binding to chitin and was thus easily separated from other proteins. Triton-X was also found to effectively elute HLL after batch binding to chitin. A further chromatographic purification step for HLL with a DEAE-Sepharose column was also effective using Triton-X for elution (Fig. 2), as most of the hemagglutination activity (HA) was recovered. Hydrophobic regions of the HLL are implicated in playing a major role in the binding interactions with both the chitin and Sepharose matrices as indicated by the efficiency of the Triton-X elution protocols presented above.

2.3. Characterization of the purified HLL

The molecular weight of the active native HLL was determined by gel filtration in the presence of Triton X-100, according to the method employed for determination of a large molecular weight human membrane bound lectin (Baenziger and Maynard, 1980). The native HLL was a very large protein of *ca* 276 kDa (Fig. 3a). The M_r obtained by SDS–PAGE analysis showed a single purified

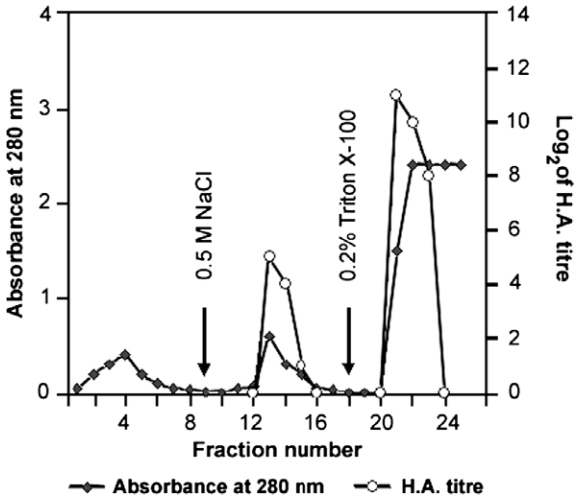

Fig. 2. Elution profile of HLL on a DEAE-Sepharose column.

HLL band of *ca* 17 kDa (Fig 3b and c). The HLL was thus unique in being a large multimeric protein comprising as many as 16 monomeric units of the 17-kDa protein. This is a major new finding, different from those found for the dimeric latex lectins previously reported in *E. Lactea* and *E. Lactea cristata* (Lynn and Clevette-Radford, 1986). The purified HLL showed a specific titre of 4.9 ng ml^{-1} against rabbit erythrocytes used in the HA assay (Table 1). The minimum concentration of HLL required for detectable hemagglutination is about 16 times lower than that, 80 ng ml^{-1}, reported for the *Hevea* bark lectin (HBL) (Wititsuwannakul et al., 1998). These large differences could be due to HLL being a much larger multimeric complex compared to that of HBL.

2.4. Factors influencing the HLL activity

Several glycoproteins tested (i.e. fetuin, asialofetuin, ovomucoid, mucin, asialomucin) were either found to inhibit the HLL-induced hemagglutination in a competitive manner. However, α_1-acid glycoprotein, soybean trypsin inhibitor and a range of various mono- or disaccharides had no inhibitory activity (Table 2). Moreover, native fetuin and mucin were more effective inhibitors of HLL activity than were their asialo counterparts. Hence molecules devoid of the sialic moiety forms of *N*-acetylneuraminyl (sialic acid) residues are probably essential and actively involved as the HLL specific ligands. Based on these results from the glycoproteins tested in the assays, HLL might be one of the sialic acid specific lectins. However, the precise carbohydrate-binding specificity domain remains to be defined and verified by further analyses. Unlike the mostly cationic basic proteins found in the lutoid vacuolar contents (B-serum) (Southorn and Yip, 1968), the membranous HLL was a neutral protein with pI of 7.2 (Fig. 4).

Reports on the Ca^{2+} requirement of lectins for their interaction with carbohydrate and binding activity are well documented (Drickamer, 1993; Drickamer and Taylor,

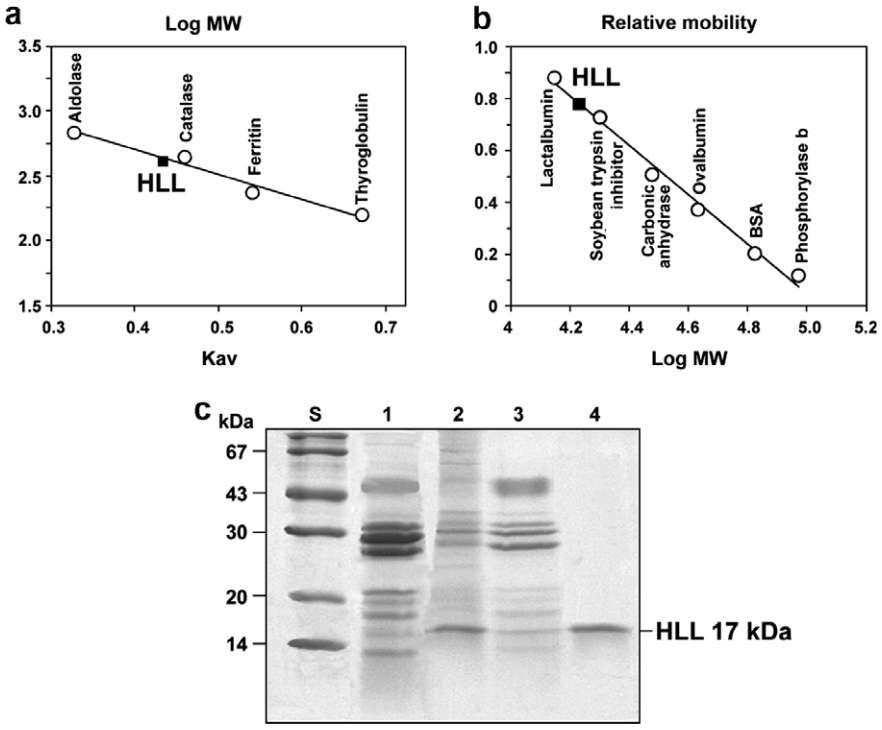

Fig. 3. Analysis of purified HLL. a and b: Molecular weight calibration of HLL by gel filtration on a Sepharose CL 6B column (a) and SDS–PAGE (b), respectively. c: SDS–PAGE. Lane 1: HLL extract (100 μg); lane 2: eluted protein (10 μg) from chitin-batch binding; lane 3: eluted protein (10 μg) from DEAE-Sepharose with 0.5 M NaCl; Lane 4: purified HLL (10 μg) eluted from DEAE-Sepharose column with 0.2% Triton X-100.

Table 1
Purification protocol of HLL

Fraction	Titre*	Specific titre** (ng ml^{-1})	Yield (%)
HLL extract	8.19×10^5	49.0	100
Chitin batch-binding	2.84×10^5	12.5	34.7
DEAE-Sepharose	2.66×10^5	4.9	32.5

* Titre is defined as the reciprocal of the lowest dilution that gives detectable agglutination of rabbit erythrocytes.
** Minimal concentration of HLL required for detectable hemagglutination.

Table 2
Carbohydrate binding specificity of HLL

Glycoprotein (mg ml^{-1})	Concentration required for 100% inhibition of agglutination of rabbit erythrocytes in the presence of HLL (1.17 μg ml^{-1})
Fetuin	0.625
Asialofetuin	1.250
Mucin	0.625
Asialomucin	2.500
Ovomucoid	2.500
Trypsin inhibitor (type II-S)	1.250

The carbohydrate binding specificity of the HLL was determinded by hapten inhibition assays using a series of mono-, di-, tri-saccharides and some glycoproteins. The following compounds were not inhibitory: glucose, galactose, mannose, fucose, arabinose (200 mM); raffinose, GlcNAc, ManNAc, GalNAc (100 mM); chitosan dimer, chitosan trimer (5 mg/ml); GlcNAc $1 \rightarrow 6$ GlcNAc, Gal $1 \rightarrow 4$ GlcNAc (2 mg/ml); 3′-*N*-Acetylneuramin-lactose, 6′-*N*-Acetylneuramin-lactose (2 mg/ml) and α_1-acid glycoprotein, type II-S trypsin inhibitor (5 mg/ml).

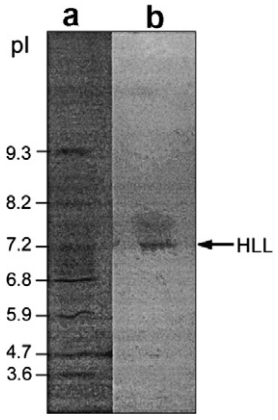

Fig. 4. pI determination of HLL. Lane a: standard markers; Lane b: purified HLL.

1993). In the case of HLL, the hemagglutination activity level was doubled with addition of low Ca^{2+} (0.5–1.5 mM) to the assay mixture (Fig. 5). This indicated that the residual Ca^{2+} present in the preparation was not sufficiently high enough to provide optimum conditions for the HA of HLL. This result was similar to those observed in a great majority of other lectin activites (Goldstein and Hayes, 1978). Surprisingly, the HLL-induced HA decreased when the concentration of the added Ca^{2+} was higher than 1.5 mM, a concentration reported to be the physiological concentration (around 1.5 mM) within the lutoid particles (d'Auzac and Jacob, 1989). The activity

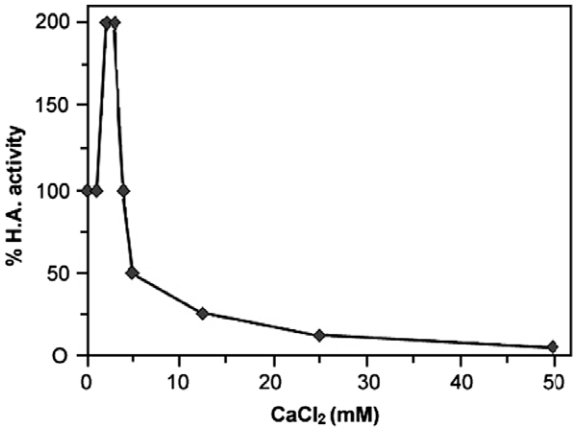

Fig. 5. Effect of $CaCl_2$ concentration on the hemagglutination activity of HLL.

was reduced by 50% at 5 mM and was almost completely inhibited at 10 mM Ca^{2+}. The biphasic nature of the Ca^{2+} effect on HLL might be an intrinsic property of its hydrophobic protein producing a specific adverse response to high Ca^{2+} ionic strength in the HA assays.

2.5. Rubber particle aggregation induced by HLL

Unlike the hemagglutination assay for HLL as presented above (Section 2.3), the assay for rubber particle (RP) aggregation is still difficult to perform and the current commonly accepted reliable method is not yet available as a routine protocol. To circumvent this problem, a novel new assay technique to monitor the RP aggregation was developed. This new assay for the HLL induced aggregation of RP was based on a specific dye binding technique

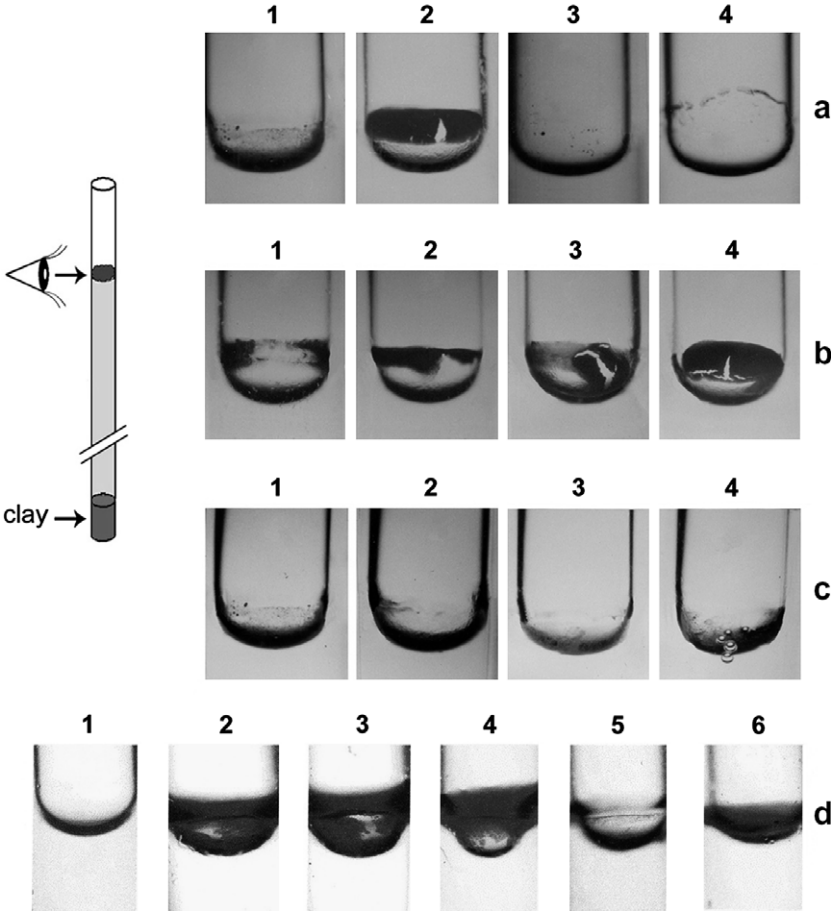

Fig. 6. RP aggregation induced by HLL. (a) Specific staining. After 30 min-incubation of RP with either control buffer (tubes1 and 3) or 1.8 μg of HLL (tubes 2 and 4), the assay mixture (50 μl) was stained with either basic Fuchsin (tubes1 and 2) or Alcian blue (tubes 3 and 4). The RP aggregates were separated by centrifugation and viewed with a light microscope. (b) Time course of HLL-induced RP aggregation. After the assay mixtures (50 μl, each) containing RP and HLL (1.8 μg) were incubated for 5, 10, 20 and 40 min as in tubes 1, 2, 3 and 4, respectively, and stained with Fuchsin. The amounts of RP aggregates obtained after centrifugation were compared. (c) Effect of various proteins other than HLL on RP aggregation. After 30 min-incubation of the RP with either control buffer (tube1), 10 μg of BSA (tube 2), 2.5 μg of fetuin (tube 3), or 8 μg of C-serum protein, the assay mixture was stained, separated by centrifuged and the amount RP aggregates were compared. (d) HLL induced aggregation of non-*Hevea* RP. HLL (1.8 μg) was incubated with either isotonic buffer (tube 1) or RP suspension prepared from the latex of *Calotropis gigantea* (tube 2); *Euphobia mili DesMoulin* (tube 3); *Ficus elastica Roxb* (tube 4); *Plumeria rubra* Linn. (tube 5); *Argyreia capitiformis* (tube 6) for 30 min. The mixtures were stained and the RP aggregates were separated and visualized.

for visualizing the stained complex using a microscopic observation. A hematocrit tube and centrifuge were employed for separating the free RP from the aggregated RP followed by examination with a microscope.

After the incubation of HLL with washed rubber particles, the RP aggregate, separated into the top layer and was specifically and intensely stained with basic Fuchsin (Fig. 6a). The Alcian blue dye that is generally used for detecting glycoproteins did not stain the aggregated complex. With a fixed amount of HLL used in the incubation, the size of RP aggregate, as evidenced by the stain intensity, increased with the incubation time (Fig. 6b). This is in addition to the intensity of the stain being dose dependent on HLL.

Lectins from other plants producing no latex (i.e. *Canavalia ensiformis*, *Cicer arietinum*, *Maackia amurensis* and *Sambucus nigra*) were either ineffective or unable to induce the RP aggregation (data not shown). Similarly, other non-lectin proteins such as bovine serum albumin (also served as the control), fresh latex C-serum proteins and fetuin (glycoprotein) were also unable to induce RP aggregation, as no staining was detected for these protein groups (Fig. 6c). These results strongly indicated a specific biological role for the endogenous latex lectin-like protein in mediating the aggregation of RP, hence the possibly that the lectin-like protein induced RP aggregation is an intrinsic physiological function. Moreover, the HLL may be a common RP agglutinin as it also induced aggregation of RP taken from several other latex producing plants (i.e. *Ficus elastica*, *Plumeria rubra*, *Euphorbia milii*, *Calotropis gigantea* and *Argyeia capitiformis*) in a similar manner to that shown for aggregation of the *Hevea* latex RP (Fig. 6d). Hence, the HLL recognition domain and the interacting glycoconjugate on RP surface receptors may be common among various different plant species.

2.6. HLL mediating latex coagulation: a proposed physiological function for latex lectin-like protein

Lutoids have been recognized for a long time and thought to play an important role in latex vessel plugging to stop the flow of latex (Southorn and Edwin, 1968; Southorn and Yip, 1968; Southorn, 1968; Milford et al., 1969; Paardekooper, 1989). The rupture or extent of lutoid bursting was shown to be inversely correlated with the rubber yield (Southorn and Yip, 1968) by shortening the duration of flow. The lutoid vacuoles are bound by a single layered fragile membrane that can be either easily damaged or ruptured by osmotic shock or the physical shear occurring naturally during the flow of latex upon tapping. Release of the lutoid membrane debris together with its vacuolar contents, that also includes Ca^{2+}, into the latex cytosolic phase had been implicated as the initiator of latex coagulation or the formation of the so called rubber coagulum (Southorn and Edwin, 1968; Southorn and Yip, 1968). The plugging of the latex vessel end was a consequence of this coagulation. The association between the damaged lutoid fragment and the RP aggregates had been demonstrated in the formation of the rubber coagulum cap over the tapping cuts or the severed latex vessel ends (Southorn, 1968). This biophysical association is now confirmed by our new and novel finding that the HLL of the lutoid membrane has a strong ability to aggregate the RP. The rupture or bursting of lutoids might ensure the exposure of the HLL, sited on the luminal membrane debris, to the RP surface glycoproteins in the presence of the concomitantly released Ca^{2+} along with other lutoid contents causing coagulation. This might eventually lead to an accumulation of the rubber coagulum into a large macromolecular complex that is sizable enough for plugging the extremity of the exuding vessel ends.

3. Conclusion remarks

Our findings have indicated the direct involvement of the lutoid membrane HLL in latex coagulation leading to the formation of a rubber coagulum from the interaction and association between the damaged lutoid membrane debris and the RP. The protein content ratios of lutoid membrane debris to rubber particles found in the rubber coagulum were from 1:54 to 1:17 (Fig. 1, tubes e–g). The process could thus lead to immobilization of the colloidal freely mobile RP. This phenomenon is different from the previous report on the rubber aggregate being cross-linked by the freely soluble small hevein protein of 4.7 kDa (Gidrol et al., 1994). That would seem to be a much less effective process, considering the very huge differences in size of RP having a maximum size distribution of 0.1 μm (Gomez and Hamzah, 1989) and the smaller hevein. Moreover, the amount of hevein required for minimum RP aggregation was observed to be much higher than that of HLL (data not shown). A physiological role for the intrinsic latex lectin-like protein in mediating RP aggregation in the rubber latex coagulation is therefore being proposed. This is a rather unique role for the lectin-like protein in latex of plants and is different from those diverse roles implicated or known for lectins in animal systems (i.e. intracellular routing of glycoproteins, cell–cell adhesion, phagocytosis) and other lectin interactions such as those proposed by (Inohara et al., 1996; Kappler et al., 1997; Ferguson et al., 1999). Further studies to purify and characterize the binding proteins recognized by or specifically interacting with HLL are thus warranted. The detailed findings are presented in the accompanying papers for this completely original long term investigative research.

4. Experimental

4.1. Chemicals

DEAE-Sepharose, glycoproteins, lectins, sugars and chitin were from Sigma, whereas SM-2 adsorbent was from Bio-Rad and Sepharose CL-4B was a product of Amer-

sham Pharmacia Biotech. All other chemicals were of reagent grade.

4.2. Plant material (collection and fractionation of latex)

Freshly tapped latex was collected in an ice-chilled beaker from regularly tapped trees of the RRIM 600 clone. The latex was fractionated by centrifugation at 49,000*g* (R_{av} 8.12 cm) for 45 min to give a floating rubber fraction, C-serum (latex cytosol) and the bottom (lutoid) fraction, respectively. The bottom fraction was separated and subjected to further treatment as described below.

4.3. Preparation of washed lutoid membrane

The bottom fraction (35 g) was washed three times by suspending in 5 volumes of Tris-buffered saline, (TBS: 50 mM Tris–HCl, pH 7.4, containing 0.9% NaCl), stirred for 30 min at 4 and recovered by centrifugation at 30,000*g* for 30 min. It was then subjected to hypotonic bursting by mixing with equal volumes of cold distilled H_2O and stirred on ice for 30 min. The lutoid membrane pellet was separated and washed three times with TBS.

4.4. Purification of Hevea latex lectin-like protein (HLL)

The washed lutoid membrane fraction was suspended in 10 volumes of buffer A (50 mM Tris–HCl pH 7.4) containing 0.2% Triton X-100 and extracted by stirring overnight at 4°. The HLL extract was separated after centrifugation at 10,000*g* for 25 min and concentrated by ultrafiltration (Amicon, M_r 10,000 cut-off). The concentrated HLL extract (5 ml) was incubated with SM2 absorbent (1:10, w/v) for 15 min to remove residual Triton X-100. It was then mixed with 10 ml of 30% (w/v) chitin solution and incubated overnight at 4°. Following adsorption, the adsorbent was extensively washed with buffer A with the bound protein eluted successively with 0.5 M NaCl and 0.2% Triton X-100 in buffer A. Fractions containing HLL were pooled, incubated with SM2 (1:10, w/v) for 15 min to remove Triton X-100 and then applied onto a DEAE-Sepharose CL-6B column (2.5 × 10 cm), previously equilibrated with buffer A. Following extensive washing, the column was successively eluted with 0.5 M NaCl and 0.2% Triton X-100 in buffer A (Fig. 1).

4.5. Hemagglutination assay of HLL

Hemagglutination assays were performed in U-well microtiter plates in a final volume of 100 μl containing 50 μl of a 2% suspension of rabbit erythrocytes, previously washed three times with TBS, and 50 μl of HLL solutions (each serially diluted with 2-fold increments). Hemagglutination was recorded visually after 1 h at room temperature, as the reciprocal of the lowest dilution giving detectable agglutination. The carbohydrate-binding specificity of the HLL was determined by inhibition assay using either the glycoproteins (fetuin, asialofetuin, mucin, asialomucin, ovomucoid, α_1-acid glycoprotein) or a series of mono-, di- or tri-saccharides. The sugars tested were glucose, galactose, mannose, fucose, arabinose, raffinose, GlcNAc, ManNAc, GalNAc, chitosan dimer, chitosan trimer, GlcNAc 1 → 6 GlcNAc, Gal 1 → 4 GlcNAc, 3′-*N*-Acetylneuramin-lactose and 6′-*N*-Acetylneuramin-lactose.

4.6. Molecular weight determination by gel filtration

The molecular weight of native HLL was determined by gel filtration on a Sepharose CL 6B column (2.5 × 85 cm) using buffer A containing 0.1% Triton X-100. Aldolase (158 kDa), catalase (240 kDa), ferritin (450 kDa) and thyroglobulin (660 kDa) were used as molecular weight standards. The molecular weight calibration was carried out according to the method described by Baenziger and Maynard (1980).

4.7. Preparation of washed rubber particles

For the latex coagulation assay, the entire rubber layer containing Moir's zone 1 and 2 (Moir, 1959) of ultracentrifuged latex was used in order to obtain sizable amount of rubber coagulum for direct visualization. The isolated rubber was thoroughly washed twice to remove any cytoplasmic protein adhering to their surface by resuspending in 10× vol by weight of TBS. The washed RP suspension was diluted to give protein concentration of 0.32 g/ml or a dry rubber content of 0.6 g/ml and used for the *in vitro* latex coagulation assay.

For the rubber particle aggregation assay, small RP were isolated from Moir's zone 2 translucent cream at the bottom of the rubber layer (facing C-serum) by skimming off and collected with a spatula. Similarly, this small RP cream was washed twice by resuspending in 10x vol. by weight of TBS. The washed small RP solution was diluted to give either a protein concentration of about 0.3 mg/ml or an absorbance at 600 nm of about 0.8–0.9 and used for the rubber particle aggregation assay.

For preparation of the non-*Hevea* latex rubber particle suspensions, incisions were made on young stems of several locally grown latex producing plants, including *Calotropis gigantea*, *Ficus elastica*, *Plumeria rubra*, *Euphorbia milii Des Moulin* or *Argyeia capitiformis*, in order to collect exuded latex into TBS. The latex was similarly washed and diluted with the same buffer to give an absorbance at 600 nm of about 0.8–0.9. All rubber particle suspensions were freshly prepared and kept on ice for use that day.

4.8. Latex coagulation assay

Three aliquots of a washed lutoid membrane suspension, containing measured amounts of total protein and dry weight contents as indicated in the text, were mixed with 4 ml of a rubber particle suspension with the total dry weight and protein content as indicated. The whole

mixture was readjusted to a final volume of 6 ml with TBS, thoroughly mixed and incubated for 30 min before being separated by centrifugation at 49,000*g* for 30 min. The increased rubber layer volumes due to latex coagulation and the disappearance of the bottom lutoid membrane fractions were visualized and compared with the controls.

4.9. Rubber particle aggregation assay

A new method was developed for the assay of rubber particle aggregation induced by HLL. The rubber particle suspension (25 μl) was mixed with 25 μl of solution containing either HLL or other proteins as indicated and incubated at room temperature for 30 min. After staining by mixing with 5 μl of basic fuchsin, 0.1% w/v, or other dye as indicated, the mixture was loaded into a hematocrit tube by means of capillary suction. The mixture-containing end was plugged by pressing it into the Seal-ease from Clay Adams Co. or modeling clay. The rubber particle aggregate that separated into the top layer after 5 min centrifugation in a micro-hematocrit centrifuge, was examined microscopically. Aggregation and autoaggregation controls were, respectively, carried out by mixing HLL with isotonic buffer and rubber particles with isotonic buffer.

4.10. Effect of $CaCl_2$ concentration

Various concentrations of $CaCl_2$ were added to the hemagglutination assay buffer as indicated. The control was carried out in the same way but without $CaCl_2$ addition.

4.11. Isoelectric focusing

Isoelectric focusing was performed with a 5% polyacrylamide gel containing 2% Biolyte 3/10 ampholytes in the Bio-Rad minigel IEF apparatus (Model 111 Mini IEF Cell). The potential difference was increased stepwise according to the manufacturer's instructions.

4.12. Polyacrylamide gel electrophoresis

SDS–PAGE was conducted as described by Laemmli (1970) with 12 % (w/v) acrylamide gels.

4.13. Protein determination

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

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