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# A rubber particle protein specific for *Hevea* latex lectin binding involved in latex coagulation ☆

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**Abstract**

In the first of this three paper series, an *in vitro* latex coagulation was shown to arise from aggregation of rubber particles (RP) and lutoid membranes. RP aggregation was shown to be induced by a specific *Hevea* latex lectin-like protein (HLL) present on the lutoid membrane. In this second paper, a binding protein (BP) ligand counterpart for HLL was identified. This RP-HLLBP, having a specific interaction, with HLL was isolated from RP and characterized. The protein was extracted from the small RP in the presence of a surfactant (0.2% Triton-X-100) and further purified to homogeneity. Purification steps included acetone precipitation, heat-treatment, and column chromatography. The presence of RP-HLLBP was monitored by its ability to compete with erythrocytes in the hemagglutination inhibition (HI) assay. The purified RP-HLLBP had an HI titre of 1.37 μg $ml^{-1}$, a p*I* value of 5.4, optimum activity at pH 5–8 and was thermostable up to 60 °C. On SDS–PAGE a single glycoprotein with $M_r$ of 24 kDa was detected while on native PAGE the major $M_r$ was about 120 kDa. The purified RP-HLLBP was shown to inhibit latex coagulation. Chitinase, but no other glycosidase tested, abolished its HI action and inhibited HLL-induced RP aggregation in a competitive dose dependent manner. This indicated the presence of, and role for, *N*-acetylglucosamine residues in the binding recognition. The *Hevea* latex lectin-like protein can thus be referred to as a *Hevea* latex lectin. Based on protein identification by peptide mass fingerprinting, the RP-HLLBP was confirmed to be the small rubber particle protein (SRPP). This work has unambiguously determined the role of an intrinsic RP glycoprotein (RP-HLLBP or SRPP) as a key component in formation of the rubber latex coagulum.


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

## 1. Introduction

Rubber particles (RP) are colloidal components present in natural rubber latex. They comprise 30–45% of the whole latex volume. The size of RP in fresh latex varies over a wide limit, from 0.02 to 3 μm (Southorn and Yip, 1968; Gomez and Hamzah, 1989) with maximum distribution of 0.1 μm (van den Tempel, 1952; Gomez and Moir, 1979). Three distinct zones with RP have been separated by means of ultracentrifugation of fresh latex (Moir, 1959). The largest RP particles are found in the uppermost Moir's zone 1 (Southorn, 1969; Yeang et al., 1995). Due to a large area occupied by zone 1, its RP diameter of 1.55 μm, as well as those as large as 3–6 μm, had been reported (Dickenson, 1969). In Moir's zone 2, the size of RP varies from 0.05 to 0.25 μm and those in Moir's zone 3 are of a lower average size (0.035–0.2 μm) (Hamzah and Gomez, 1982). Two major RP proteins of 14.6 and 24 kDa had been classified as proteolipids and identified in washed RP obtained from ultracentrifuged fresh latex

☆ Part 2 in the series 'Proteins specifically involved in *Hevea* rubber particle aggregation and latex coagulation'.

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(Hasma, 1987; Wititsuwannakul et al., 2004). The genes encoding these two major proteins were found to be most abundant in the analysis of the *Hevea* lacticifer transcriptome (Ko and Chow, 2003). A deduced amino acid sequence obtained from a full-length c-DNA encoding the 24 kDa protein was shown to have a high homology to that of the 14.6 kDa protein (Oh et al., 1999). Both the 14.6 kDa (Hev b1), a first latex allergen reported in general population, and 24 kDa (Hev b3) proteins are major latex allergens in patients with spina bifida (Czuppon et al., 1993; Alenius et al., 1993; Yeang et al., 1996). The major rubber particles protein of 24 kDa has been suggested to be tightly associated with small rubber particles and also involved in rubber biosynthesis (Oh et al., 1999). A later study on micromorphology and *Hevea* rubber particle protein characterization has indicated that the 24 kDa core protein might not be the protein necessary for rubber biosynthesis (Singh et al., 2003). The exact role for the RP 24 kDa protein remains to be verified and discerned upon further careful studies, so the ambiguity could be resolved.

In this paper, a protein present on the smaller RP (the rubber layer zone 2 of centrifuged fresh latex) was purified and shown to be a specific binding protein for the *Hevea* latex lectin as well as being an inhibitor of latex coagulation. This binding protein on the RP surface serves as a specific receptor for the latex lectin interaction and sequentially mediates the latex coagulation.

## 2. Results and discussion

In our previous paper, the aggregation of small RP was shown to be induced by HLL (Wititsuwannakul et al., 2008). Although both the 14 and 24 kDa RP proteins were detected by SDS–PAGE in the Triton X-100 extracts of either large (Moir's zone 1) or small (Moir's zone 2) RP, the 24 kDa protein was found to be more abundant than the 14 kDa (Fig. 1) in the small RP. This was in contrast to the large RP where the 14 kDa was a dominant protein (data not shown). The washed small RP were, therefore, used for the extraction of the RP bound proteins.

### 2.1. Purification of a rubber particle surface protein as a HLL binding protein

RP bound protein was extracted from the washed small RP with 0.2% Triton X-100 present in the extracting solution mixture. The procedure used for successfully extracting the RP protein is similar to that employed for extracting either hydrophobic proteins or membrane-bound proteins (Barondes, 1986). The RP extracted soluble protein was shown to interact strongly with the *Hevea* latex lectin (HLL) as a specific binding protein (BP) and was thus designated as a HLL binding protein (HLLBP). In the previous report (Wititsuwannakul et al., 2008) the HLL was shown to cause either aggregation or agglutination of RP. Consequently, because washed RP was the source of the lectin binding protein, the RP protein was thus designated as RP-HLLBP. The crude extracted RP-HLLBP prior to its purification showed strong inhibition of the HLL-mediated rabbit erythrocyte hemagglutination. It was further purified to homogeneity by the purification protocol described in the Section 4. This included acetone precipitation, followed by heat-treatment and chromatographic separation using Sepharose 6B (Fig. 2) and DEAE-Sephacel (Fig. 3) columns, respectively. The cytochrome P450 protein of 53 kDa had earlier been reported as the major rubber particle protein in guayule (Pan et al., 1995). However, in our analysis of the *Hevea* RP proteins by SDS–PAGE with acetone precipitation of the RP solubilized proteins showed mainly two major bands of about 14 and 24 kDa proteins (Fig. 1). They were considered as the intrinsic proteins of *Hevea* RP with a few other

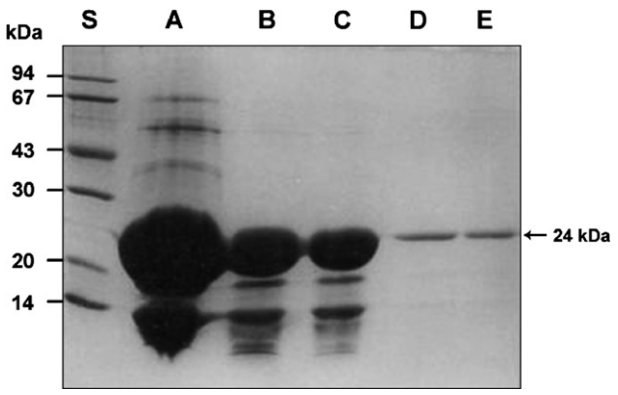


Fig. 1. SDS–PAGE analysis of the purified RP-HLLBP. The gel was stained with Coomassie Brilliant Blue R250. Lane S: standard proteins; lane A: crude RP protein extract obtained after acetone precipitation of the washed rubber particle protein extract; lane B and C: as in A but after further heat treatment; lane D and E: purified RP-HLLBP from the DEAE-Sephacel peak fraction.

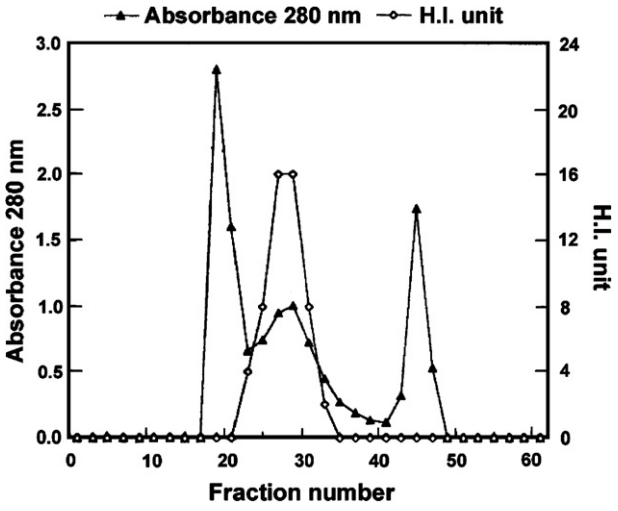


Fig. 2. Chromatographic profile of partially purified RP-HLLBP on a Sepharose 6B column. Acetone precipitated protein from a washed rubber particle protein extract was subjected to heat treatment before being loaded onto the column.

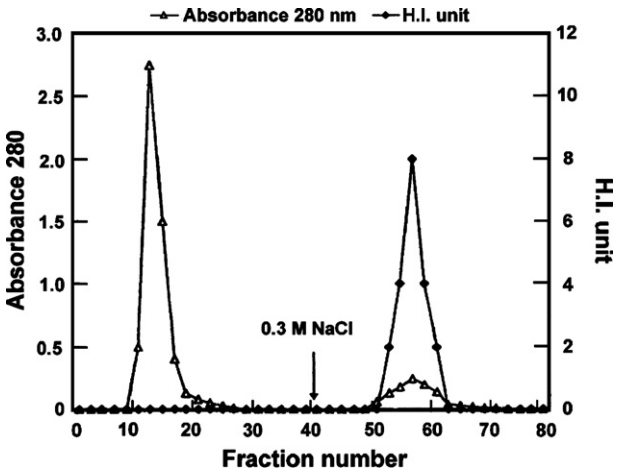


Fig. 3. Chromatographic profile of pooled active fractions from the Sepharose 6 B column on a DEAE-Sephacel column.

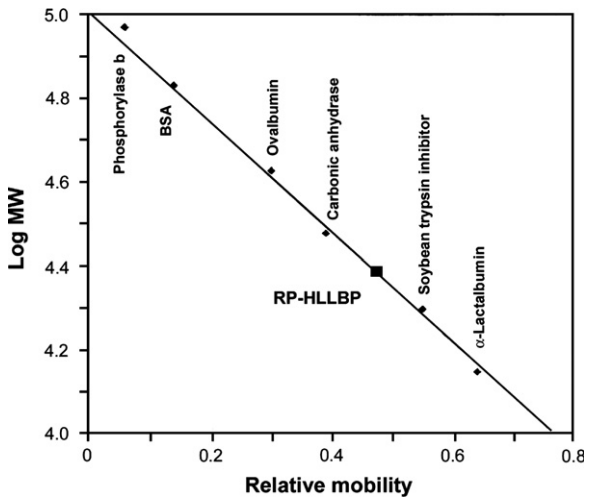


Fig. 5. Calibration curve for subunit molecular weight determination of RP-HLLBP on SDS–PAGE.

minor protein bands that likely resulted from association of the tightly bound bottom fraction membrane as earlier described (Wititsuwannakul et al., 2004). These extra and other contaminating proteins with higher molecular weight were sensitive to heat-treatment, unlike the two major 14 and 24 kDa proteins that are more heat stable. The purified RP-HLLBP, obtained after the ion exchange chromatography, showed a single protein band of 24 kDa by SDS–PAGE (Figs. 1, 4 and 5) and had a specific HI titre of 1.37 μg $ml^{-1}$ (Table 1).

Table 1
Purification protocol of RP-HLLBP

| Step | Total activity (HI titre unit[a]) | Total protein (mg) | Specific HI titre[b] (μg $ml^{-1}$) | Yield (%) |
|---|---|---|---|---|
| Acetone precipitate | $1.79 \times 10^5$ | 574 | 3.20 | 100 |
| Heat treatment | $8.32 \times 10^4$ | 241 | 2.89 | 46 |
| Sepharose 6B | $1.02 \times 10^5$ | 170 | 1.75 | 57 |
| DEAE-Sephacel | $1.60 \times 10^4$ | 22 | 1.37 | 9 |

[a] Hemagglutination inhibition (HI) titre unit is defined as the reciprocal of the lowest dilution that gives detectable inhibition of agglutination of rabbit erythrocytes.
[b] Minimal concentration of RP-HLLBP required for detectable HI.

### 2.2. Characterization of the RP-HLLBP properties and interactions

The purified RP-HLLBP produced a single band ca. 24 kDa on SDS–PAGE (Figs. 1 and 4) and major (ca. 120 kDa) as well as minor (ca. 130 kDa) bands on the non-denaturing native PAGE (Figs. 4 and 6). This result indicated that the native RP-HLLBP was a multimeric protein, either pentameric (major) or hexameric (minor)

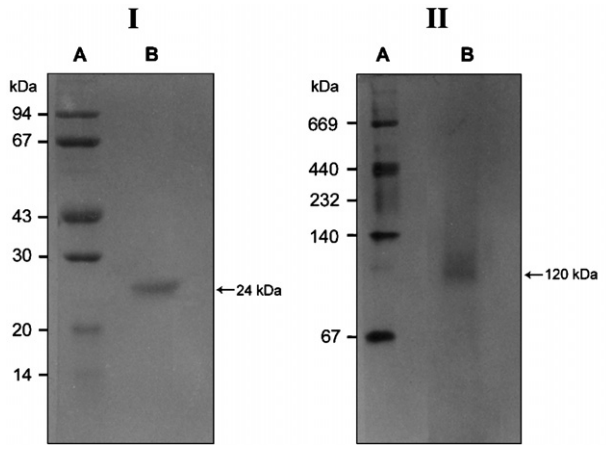


Fig. 4. SDS–PAGE (I) and native-PAGE (II) of purified RP-HLLBP, stained with Coomassie Brilliant Blue R250. Lane A: standard protein markers; Lane B: purified RP-HLLBP (30 μg).

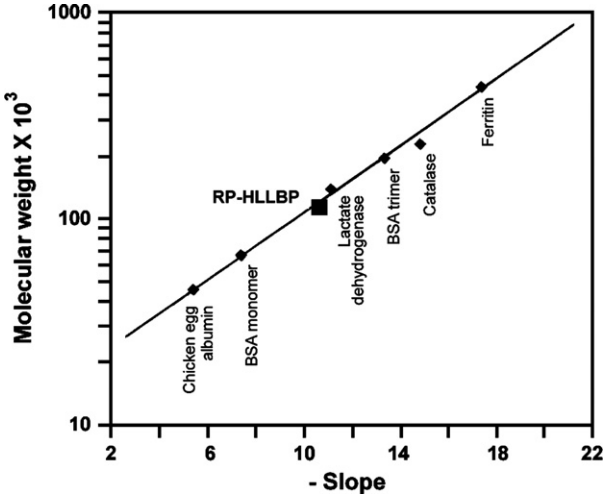


Fig. 6. Calibration curve of standard marker proteins for native molecular weight determination of RP-HLLBP on native-PAGE.

depending on the affinity of subunit association. The 24-kDa protein had been previously identified as an RP associated intrinsic protein (Wititsuwannakul et al., 2004), but with no assigned function. The findings of RP-HLLBP activity indicated a possible role for this protein in latex

coagulation. Based on protein identification by peptide mass fingerprinting data (Fig. 7), this 24 kDa protein is confirmed to be the same as that previously identified (Oh et al., 1999) as SRPP and as the Hev b 3 latex allergen (Yeang et al., 1996, 1998). However, these reports did not provide any convincing evidence for a specific role. Our finding of RP-HLLBP activity might resolve this issue (see Table 2).

The RP-HLLBP is an acidic protein. Isoelectric focusing resolved a single major component (p*I* ca. 5.2), and a minor component (p*I* ca. 6.0) (Fig. 8). These p*I* values obtained are in agreement with pH stability ranges of 5–8 for the active RP-HLLBP (Fig. 9). The heterogeneity of the (native) intact protein on isoelectric focusing may reflect a tendency for associating among itself into either pentameric (major) or hexameric (minor) forms, similar to those observed under the non-denaturing PAGE (Fig. 4). The native active form of RP-HLLBP was heat-stable up to 60 °C. Above 60 °C up to 80 °C, the HI activity dropped sharply to 50% remaining and at 90 °C and 100 °C only 20% remained (Fig. 10). Among all the proteins extracted from RP by Triton X-100, the 24-kDa RP-HLLBP was the only glycoprotein. On SDS–PAGE analyses of the separated proteins, the 24-kDa protein band was the only one that was stained by PAS and Alcian blue dyes (Fig. 11).

The purified 24-kDa glycoprotein, RP-HLLBP, had a 4.25% (w/w) neutral sugar content as determined by the phenol–sulfuric acid method that is commonly employed for neutral sugar analyses. Treatment of the glycoprotein

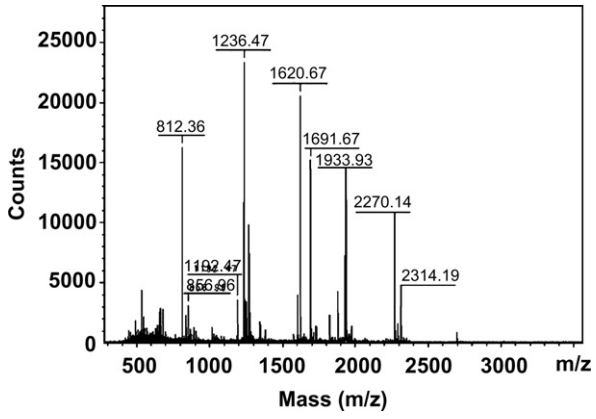


Fig. 7. RP-HLLBP tryptic digest mass spectrum from peptide mass fingerprinting analysis by MALDI -TOF mass spectrometry.

Table 2
Effect of glycosidase treatments on HI activity of RP-HLLBP

| Treatment | HI activity (% control) |
|---|---|
| 1. RP-HLLBP[a] (control) | 100 |
| 2. As 1 + galactosidase (30 U) | 100 |
| 3. As 1 + glucosidase (30 U) | 100 |
| 4. As 1 + neuraminidase (0.15 U) | 100 |
| 5. As 1 + chitinase (0.15 U) | 0 |

[a] The amount of RP-HLLBP was 40 μg in a total assay volume of 30 μl.

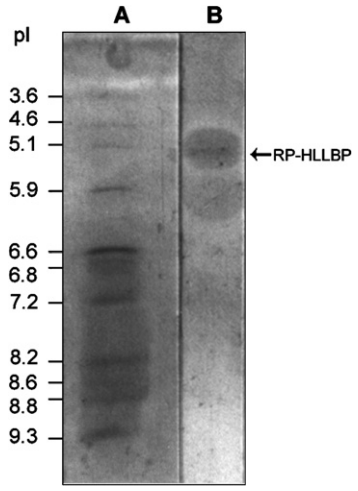


Fig. 8. Isoelectric focusing of RP-HLLBP. The gel was stained with Coomassie Brilliant Blue R 250.

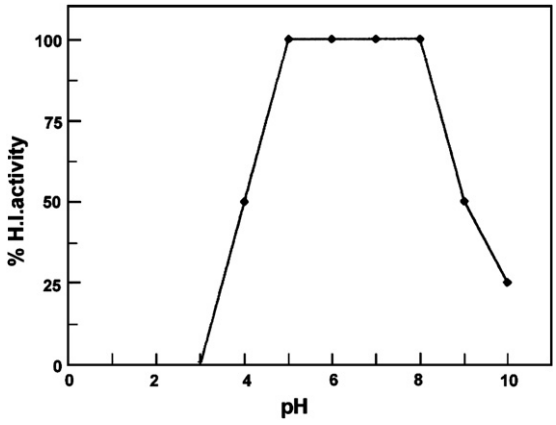


Fig. 9. pH stability of RP-HLLBP.

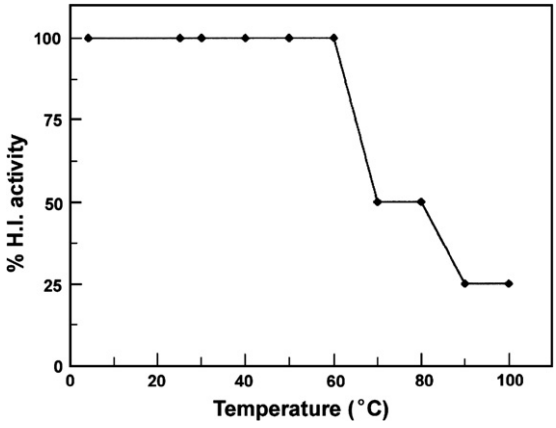


Fig. 10. Temperature stability of RP-HLLBP determined by the HI activity.

with different glycosidase or glycohydrolase enzymes (galactosidase, glucosidase, neuraminidase and chitinase) followed by assaying for remaining HI activity showed that only chitinase completely abolished HI activity. The

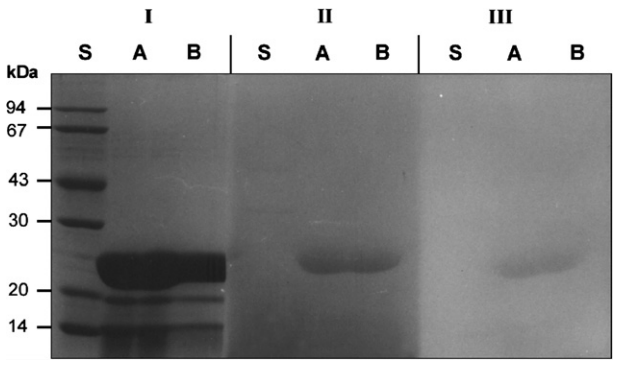


Fig. 11. SDS–PAGE of RP-HLLBP, stained with Coomasie Brilliant Blue R250 (I), PAS (II) and Acian blue reagent (III). Lane S: standard proteins; Lane A and B contained 150 and 100 μg of partially purified RP-HLLBP obtained after heat-treatment.

decrease in HI activity was proportional to either the concentration or units of the chitinase enzyme levels applied for treatment (Fig. 12). Hence, the RP-HLLBP contains *N*-acetylglucosamine residues involved in the binding recognitions by the *Hevea* latex lectin-like protein which can now be referred to as *Hevea* latex lectin. The purified RP-HLLBP has a specific HI titre of 1.37 μg $ml^{-1}$ in the specific HA inhibition assays. The minimum concentration of RP-HLLBP required for detectable hemagglutination inhibition is much lower than those reported for glycoproteins from non-latex origin (Wititsuwannakul et al., 2008). Perhaps this is another illustration of the high specificity that can exist in biologically important specific recognition phenomena and biochemical specific interactions. Normally these specific reactions between a ligand and its receptor are signaling events leading to some specific cellular response. In this case it would seem that the intrinsic RP-HLLBP ligand reacts with its natural latex lectin receptor to facilitate latex coagulation and plugging of the latex vessels.

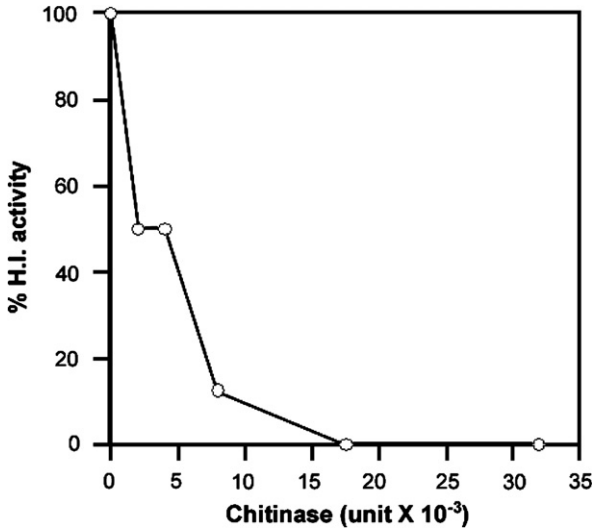


Fig. 12. Effect of chitinase on RP-HLLBP activity.

### 2.3. Inhibition of latex coagulation by purified RP-HLLBP

A complete latex coagulation process occurred after mixing appropriate amounts of washed lutoid membrane with RP, as indicated (Fig. 13, tube C). The protein content ratio, ca. 1:54, of lutoid membrane debris to RP found in the rubber coagulum were similarly shown in earlier report (Wititsuwannakul et al., 2008). This indicates a single lutoid membrane complexing several rubber particles. Accordingly, from an electron microscopic study of latex vessel in *Hevea* bark, the destabilized lutoids had been shown to have numerous small rubber particles zone 2 variety adhering to their surfaces (Yeang et al., 1995). All the lutoid membrane debris disappeared from the bottom fraction due to their rapid aggregation with the RP. However, a prior incubation of the lutoid membrane with purified RP-HLLBP was found to reduce the extent of their ability in aggregating the RP. The higher the amounts of the soluble RP-HLLBP added to the pre-incubation mixtures, the higher were the remaining amounts of unaggregated lutoid membrane in the bottom fraction (Fig. 13, tubes D–F). These results indicate that HLLBP, in either soluble or surface-bound forms was specifically recognized by the lutoid membrane HLL while the surface-bound HLLBP was necessary for the latex coagulation.

### 2.4. RP-HLLBP as the latex coagulating factor

The assay developed to monitor the HLL induced aggregation of RP (Fuchsin staining) (Wititsuwannakul et al., 2008) was used to test the possibility that the specific function of RP-HLLBP, the lectin binding protein, was associated with latex coagulation. HLL was preincubated with various amounts of purified RP-HLLBP followed by addition of the washed small RP. RP-HLLBP inhibited the HLL-induced RP aggregation in a dose dependent manner (Fig. 14). The amount of stained RP aggregate without the RP-HLLBP was sequentially reduced as the BP level increased until it was completely abolished by the BP. However, when RP-HLLBP was pretreated with chitinase, but not by other glycosidase enzymes, there was no reduction of the stained RP aggregate (Fig. 15). These results indi-

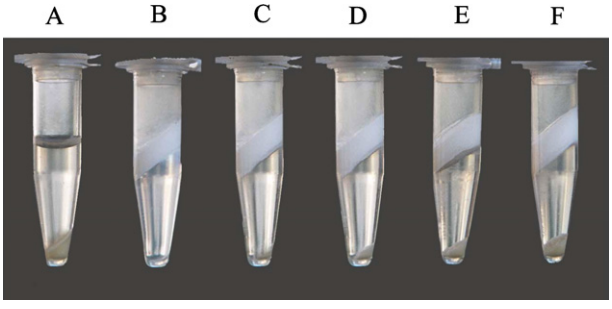


Fig. 13. Centrifugation of suspensions A–H, 0.5 ml each. A: washed lutoid membrane, containing 1.2 mg protein; B: washed rubber particle, with 64 mg protein, C: A + B; D–F: (A + RP-HLLBP) + B, the total amounts of purified RP-HLLBP in D–F were 15, 45, and 90 μg, respectively.

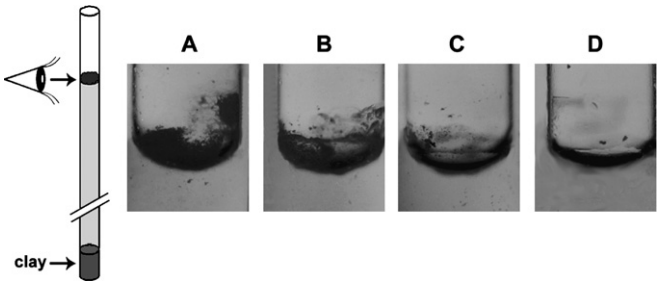


Fig. 14. Inhibitory effect of RP-HLLBP on HLL-induced rubber particle aggregation. The HLL (2.5 μg) was preincubated with either assay buffer only (A) or buffer solution containing 5 (B), 20 (C) or 40 (D) μg of RP-HLLBP before testing in the rubber particle aggregation assay as described Section 4.

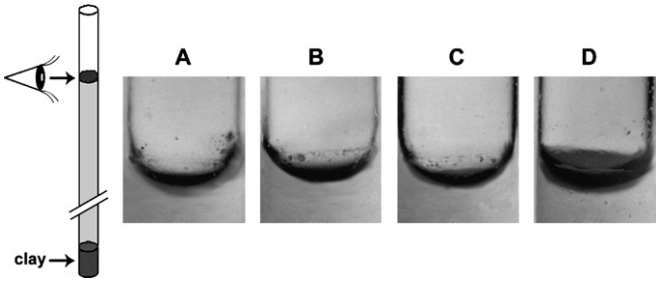


Fig. 15. Effect of various glycosidases on the inhibition of HLL-induced rubber particle aggregation by RP-HLLBP. The RP-HLLBP (40 μg) was preincubated with either (A) galactosidase, 15 U; (B) glucosidase, 1.5 U; (C) neuraminidase, 0.075 U or (D) chitinase, 0.075 U, before testing its inhibitory effect on HLL-induced rubber particle aggregation as described Section 4.

cated that *N*-acetylglucosamine on the RP-HLLBP was actively involved in the specific binding recognition. A similar role has been postulated for the *N*-acetylglucosamine sugar on the RP glycoprotein receptor, in mediating polyvalent bridging between hevein and RP (Gidrol et al., 1994). Although chitinase was demonstrated to have a negative effect on the HLL-induced RP aggregation, this effect was considerably reduced in the presence of $Ca^{2+}$, previously shown to promote the HLL agglutinating activity (Wititsuwannakul et al., 2008). The chitinase and $Ca^{2+}$ are both lutoid vacuolar constituents and are concurrently released upon bursting of the lutoids. Hence, the contents and levels of the exposed HLL binding sites on the ruptured lutoid membrane might be key limiting factors in promoting latex coagulation. This agrees well with the reports of a high correlation between lutoid bursting and the latex vessel plugging indexes (Yeang and Paranjothy, 1982; d'Auzac, 1989). These findings indicate that the small RP with the intrinsic 24-kDa surface glycoprotein receptor acts as a coagulating factor in the rubber latex coagulation process.

## 3. Concluding remarks

This proposed physiological function for the RP-HLLBP or SRPP is strongly substantiated by previous findings on the tight association of the 24-kDa protein with the small RP surface (Oh et al., 1999). Therefore, the logic of these findings is that the highly abundant small RP, less than 0.36 μm in size and accounting for ca. 95% of the total RP population in *Hevea* latex (van den Tempel, 1952; Gomez and Hamzah, 1989), has an important role in the latex coagulation process.

## 4. Experimental

### 4.1. Chemicals

DEAE-Sephacel, glycoproteins, sugars and chitinase were from Sigma. Biogel P-300 was from Bio-Rad (USA). All other chemicals were of reagent grade.

### 4.2. Purification of RP-HLLBP

The washed RP suspension, prepared as previously described (Wititsuwannakul et al., 2008), was extracted with 5× vol. of 0.2% Triton X-100 detergent. The mixture was stirred overnight at 4 °C and centrifuged to obtain the supernatant. Two volumes of cold acetone was added, mixed well and kept on ice for 10 min. The solution was then centrifuged at 10,000*g* for 10 min at 4 °C. The acetone precipitate was dissolved in 50 mM Tris–HCl, pH 7.4 (buffer A) and again centrifuged at 40,000*g* for 10 min to separate the supernatant. The first step for purifying the RP-HLLBP was to heat the resuspended acetone precipitated solution at 70 °C for 5 min followed by immediately placing it in an ice bath and centrifuging at 40,000*g* for 10 min to remove the precipitate. The supernatant was concentrated and subjected to further purification on a Sepharose 6 B column (1.4 × 70 cm) previously preequilibrated with buffer A at a flow rate of 12 ml/h at 4 °C. After loading the sample, the column was eluted with the same buffer. Fractions of 2 ml were collected and their absorbance measured at 280 nm. An HI assay was performed in all fractions. The fractions containing HI activity were pooled, concentrated and further purified on a DEAE-Sephacel column (1.8 × 8 cm), preequilibrated with buffer A. After loading the sample, the column was washed with the same buffer until the absorbance at 280 was below 0.005. The column was then eluted using the same buffer containing 0.3 M NaCl. The fractions containing high HI activity were pooled, desalted and concentrated for further characterization studies.

### 4.3. Assay of hemagglutination inhibition (HI) by RP-HLLBP

The activity of RP-HLLBP activity was measured by its ability to inhibit hemagglutination induced by HLL. Each 25 μl of RP-HLLBP sample was 2-fold serially diluted with hemagglutination buffer (TBS), containing 0.9% NaCl in 50 mM Tris–HCl buffer, in a microtitre U plate. This was

followed by the addition of HLL solution (25 μl) that possessed a titre of 4 hemagglutination units. The solution was mixed and incubated at room temperature for 20 min before the addition of 50 μl of a 2% (v/v) rabbit erythrocyte suspension into each well. Hemagglutination was recorded after incubation for 1 h at room temperature. The minimum concentration of inhibitors that caused 100% inhibition of hemagglutination activity of the HLL was calculated. The inhibition activity was expressed in term of hemagglutination inhibition (HI) unit or titre.

### 4.4. Effect of glycosidases on HI activity of RP-HLLBP

7.5 μl aliquots each containing 40 μg of partially purified RP-HLLBP were incubated with 7.5 μl of glycosidase enzymes with various activity units (galactosidase, 30 U; glucosidase, 30 U; neuraminidase, 0.15 U and chitinase 0.15 U or as indicated) for 30 min at room temperature. After incubation, the HI activity of RP-HLLBP was determined in each reaction mixture as described above (see the previous section).

### 4.5. Effect of RP-HLLBP on HLL-induced rubber particle aggregation

A solution (15 μl) containing 2.5 μg of HLL, isolated and purified as described previously (Wititsuwannakul et al., Paper #1 in series), was incubated with 15 μl of various amounts of the RP-HLLBP from 5 to 40 μg. Incubation without the RP-HLLBP served as the control. After incubation for 30 min, the rubber particle aggregation assay was performed by adding 30 μl of rubber particle suspension to the mixture. The rubber aggregate formed was separated and observed as described previously (Wititsuwannakul et al., 2008).

### 4.6. Effect of glycosidases treated RP-HLLBP on HLL-induced RP aggregation

Aliquots (30 μl) containing partially purified RP-HLLBP, obtained after pretreatment with various glycosidases (galactosidase, 15 U; glucosidase, 1.5 U; neuraminidase, 0.075 U and chitinase, 0.075 U) as described above were incubated with 2.5 μg of HLL for 30 min. After the incubation, a washed RP suspension (30 μl) was added to the assay mixture and incubation was continued. The complete assay mixture was then stained with Fuchsin. The rubber aggregate formed was separated and observed as described previously (Wititsuwannakul et al., 2008).

### 4.7. Effect of pH and temperature

The effect of temperature on the HI activity of RP-HLLBP was determined by incubating a RP-HLLBP sample at various pHs (for 1 h) or temperature (for 30 min) as indicated. The mixtures were then adjusted back to pH 7.4 or 4 °C and assayed for HI activity.

### 4.8. Carbohydrate determination of RP-HLLBP

The neutral sugar content of the RP-HLLBP was determined in a scaled down version of the phenol–sulfuric acid method of Dubois et al. (1956).

### 4.9. Polyacrylamide gel electrophoresis

PAGE was performed either in the presence or absence of SDS by the method of Laemmli (1970) and stained as indicated.

### 4.10. Glycoprotein staining

SDS–PAGE of partially purified RP-HLLBP was subjected to PAS staining according to the method of Zacharius et al. (1969) and Alcian blue staining as described by Wardi and Michos (1972).

### 4.11. Polyacrylamide gel isoelectric focusing

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

### 4.12. Latex coagulation inhibition assay

Fifty microliter aliquots each containing a washed lutoid membrane suspension with a total protein content of 1.2 mg, prepared as previously described (Wititsuwannakul et al., 2008), were mixed with 50–200 μl of the purified RP-HLLBP with various amounts of protein as indicated and incubated for 1 h at room temperature. Two hundred microliter of a washed RP suspension with protein content of 64 mg or dry rubber content of 120 mg, prepared as previously described (Wititsuwannakul et al., 2008), was then added. The final volume of the mixture was maintained at 500 μl by adding TBS buffer and thoroughly mixed. After additional 30 min incubation, the mixture was separated at 10,000 rpm for 20 min in a microcentrifuge. The amounts of the decrease in the size of rubber coagulum and increase in the size of remaining bottom lutoid membrane obtained after the final incubation were visualized and compared with the latex coagulation control tube. A mixture containing 50 μl of washed lutoid membrane, 200 μl of washed RP suspensions and 250 μl of TBS was used in the latex coagulation assay control. The washed RP assay control contains 200 μl RP suspension and 300 μl TBS and the washed lutoid membrane control, 50 μl lutoid membrane suspension and 450 μl TBS.

### 4.13. Protein identification by mass spectrometry

The electrophoretic band corresponding to the 24 kDa RP-HLLBP was excised and digested with trypsin. A pep-

tide mass fingerprint (PMF) was produced by analysing the digested protein with a Bruker Biflex III MALDI-TOF mass spectrometer. The resulting PMF was compared with the calculated masses of all tryptic peptides that can be theoretically produced from the sequences corresponding to all *Hevea brasiliensis* proteins in SWISS-PROT and NCBInr public protein databases. The protein that yielded the best match between the theoretical and experimental mass values was identified to be a small rubber particle protein. The MALDI-MS analysis was carried out, under a requested service, at The University of Minnesota Center for Mass Spectrometry and Proteomics.

### 4.14. 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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