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Hevea latex lectin binding protein in C-serum as an anti-latex coagulating factor and its role in a proposed new model for latex coagulation ☆

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Abstract

A distinct protein specifically recognized by its strong interaction with *Hevea* latex lectin (HLL) was detected in the aqueous C-serum fraction of centrifuged fresh latex. This C-serum lectin binding protein (CS-HLLBP) exhibited strong inhibition of HLL-induced hem-agglutination. The CS-HLLBP was purified to homogeneity by a protocol that included ammonium sulfate fractionation, size exclusion and ion exchange chromatography. The purified CS-HLLBP had a specific HI titer of 0.23 μg ml^{-1}. Its M_rs analyzed by SDS–PAGE was *ca.* 40 kDa and that by gel filtration was *ca.* 204 kDa. It has a p*I* value of 4.7, an optimum activity between pH 6 and10 and was heat stable up to 50 °C. The HI activity of CS-HLLBP was abolished upon treatment with chitinase. The CS-HLLBP inhibited HLL-induced rubber particle aggregation in a dose dependent manner. A highly positive correlation between CS-HLLBP activity and rubber yield per tapping was found. The correlations for fresh latex ($r = 0.98$, $P < 0.01$) and dry rubber ($r = 0.95$, $P < 0.01$) were both highly significant. This indicated that the CS-HLLBP might be used as a reliable marker for the mass screening of young seedlings to identify and select clones with potential to be superior producers of rubber. A latex anti-coagulating role of the CS-HLLBP is proposed. The findings described in this 3 paper series have been used to propose a new model of rubber latex coagulation that logically describes roles for the newly characterized latex lectin and the two lectin binding proteins.

Keywords: *Hevea brasiliensis*; Euphorbiaceae; Rubber latex; C-serum; Lectin; Lectin binding protein; α-Globulin; Latex flow; Anti-coagulating factor; Latex coagulation

1. Introduction

The C-serum fraction of centrifuged fresh latex represents the aqueous phase of the laticiferous cytoplasmic contents, and can be considered to be the metabolically active fraction of the latex cytosol. Glycolytic enzymes (d'Auzac and Jacob, 1969) and other common cytosolic enzymes, including those of the isoprenoid pathway (Wititsuwannakul et al., 1990; Suvachitanont and Wititsuwannakul, 1995; Koyama et al., 1996) have been detected in the C-serum. These findings have indicated that the C-serum is active in a number of different metabolic processes. An active involvement of the C-serum in rubber biosynthesis has been noted (Tangpakdee et al., 1997), as has the importance of a heat stable calcium binding protein (calmodulin) in regulating these different interrelated

☆ Part 3 in the series 'Proteins specifically involved in *Hevea* rubber particle aggregation and latex coagulation'.

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metabolic processes. A purified calmodulin from C-serum was clearly shown to activate HMG-CoA reductase in the bottom (lutoid) or membrane fraction (Wititsuwannakul et al., 1990). The composition of the latex cytosol C-serum has been reviewed (d'Auzac and Jacob, 1989; Wititsuwannakul and Wititsuwannakul, 2001). The presence of many high molecular weight compounds, low-molecular weight organic solutes and mineral elements has been well documented. The high molecular weight compounds in C-serum are mainly proteins and nucleic acids. The distribution of proteins in whole latex is approximately 20% in the rubber phase, 20% in the bottom fraction, and 60% in the C-serum (Archer and Sekhar, 1955; Archer et al., 1963; Wititsuwannakul and Wititsuwannakul, 2001). Gel electrophoresis analysis of C-serum (Tata and Edwin, 1970) has indicated a large number of different proteins at varying concentrations. One C-serum protein that is present at a highest level is α-globulin. The α-globulin has a p*I* of 4.55, the same as that of latex. Due to its ease of adsorption onto rubber particles there has been a suggestion that it could play a key role in contributing to the latex and rubber particles' colloidal stability (Archer et al., 1963; Archer and Cockbain, 1955).

In this paper, the C-serum is shown to contain another *Hevea* latex lectin (HLL) binding protein with properties in many way similar to those of the previously isolated surface-bound protein on rubber particles (Wititsuwannakul et al., paper #2 in this series). This study demonstrates that this protein has an anti-coagulating role that is important physiologically for maintaining the colloidal stability by preventing the coagulation of the latex. A new model for rubber latex coagulation is presented, based on the balanced interactions between the HLL and the two binding proteins that are dependent on the stoichiometric ratios for the extent and selectivity of the dynamic variable real time interactions.

2. Results and discussion

In the preceding part of our studies on the HLL, the glycoprotein located on the small rubber particle (RP) surface was specifically recognized by and interacted with the HLL also previously characterized (Wititsuwannakul et al., 2007, papers #2 in this series). In this report, a different HLL binding protein was identified in the C-serum fraction of centrifuged fresh latex. This new C-serum HLL binding protein had unique properties, characterized in this report, in addition to it being a soluble protein located in the C-serum.

2.1. Purification of the soluble HLL binding protein in the latex C-serum

The protein purified from the latex C-serum bound strongly to HLL and strongly inhibited HLL-mediated hemagglutination. The amount of lectin binding protein and its specific activity was monitored by the level of HI titres in the assays. Purification of the C-serum HLL binding protein (CS-HLLBP) was achieved by a protocol employing fractionation with ammonium sulfate, gel filtration (Bio-gel P-300) and ion exchange (DEAE-Sephacel) chromatography (see Table 1). The first protein peak eluted from the Bio-gel had a high HI titre but only a small amount of protein (Fig. 1). Fractionation on DEAE-Sephacel produced a symmetrical protein peak after elution with buffer A plus NaCl, that coincided exactly with the only fraction with a high HI titre (Fig. 2). The purified protein produced a single band upon SDS–PAGE analysis (Fig. 3), with an M_r of *ca* 40 kDa . This protein also appeared as a major band in the 70–85% ammonium sulfate fraction and was the major band from the Biogel fraction with the high HI activity (Fig. 3). The native form of the HI active protein had an M_r of *ca* 204 kDa as determined by gel filtration. This indicated that it was a multimeric form of the 40 kDa protein subunits. This property is similar to that of the RP-HLLBP previously identified and characterized (Wititsuwannakul et al., paper #2 in this series) but the subunits and their multimeric form have different M_r values and other properties are different.

Table 1
Purification protocol of CS-HLLBP

Step	HI titre[a] (titre)	Specific HI titre[b] (μg ml^{-1})	Yield (%)
$(NH_4)_2SO_4$ precipitate	7.68×10^3	3.20	100
Bio Gel P-300	3.20×10^3	0.48	42
DEAE-Sephacel	2.56×10^3	0.23	33

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

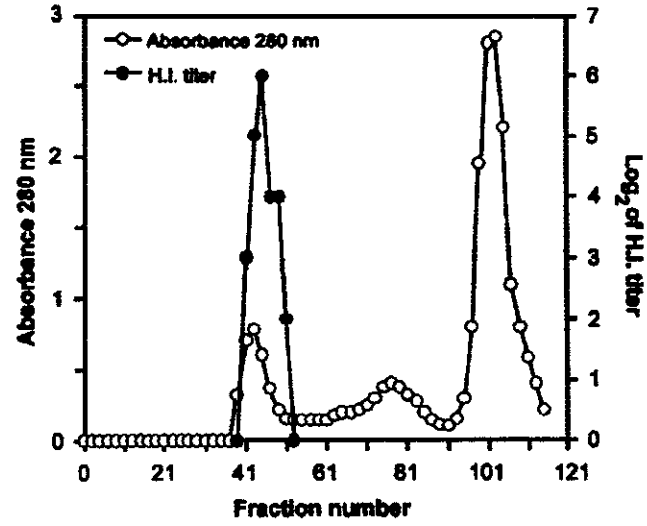

Fig. 1. Chromatographic profile of crude CS-HLLBP on a Bio-gel P-300 column obtained after $(NH)_2SO_4$ fractionation.

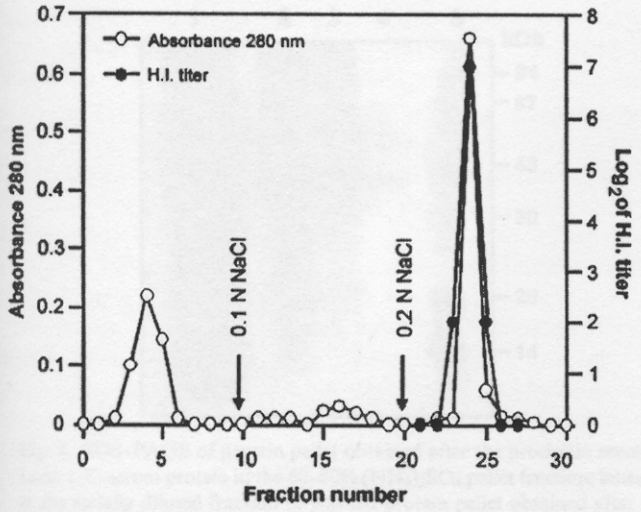

Fig. 2. Chromatographic profile of pooled active fractions of CS-HLLBP from the Biogel P-300 column on a DEAE-Sephacel column. The column was eluted by the stepwise increase of NaCl as indicated.

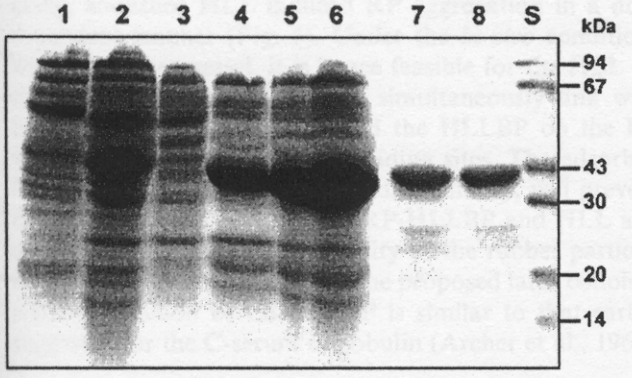

Fig. 3. SDS–PAGE analysis of the purified CS-HLLBP. Lane S: standard proteins; lane 1: C-serum proteins in 40–75%, lane 2: 70–85%, lane 3: 0–40% $(NH_4)_2SO_4$ pellet fractions; Lane 4–6: pooled peak fractions from BioGel P-300 column with 50, 75 and 100 μg protein, respectively; Lanes 7 and 8 : purified CS-HLLBP (20 μg, each) from eluted peak fraction on DEAE-Sephacel column.

2.2. Characterization and properties of the C-serum-HLL binding protein

The purified CS-HLLBP is an acidic protein with p*I* value of *ca* 4.7 similar to that reported for α-globulin (Archer et al., 1963). SDS–PAGE analyses (Fig. 3) showed that it was the most abundant C-serum protein. Archer et al. (1963) had previously identified α-globulin as the most abundant C-serum protein. The native protein was heat stable up to 50 °C as monitored by its remaining HI titres between 4 and 90 °C. Above 50 °C, the remaining HI titre was reduced by 50% up to 80 °C. These results indicate that above 50 °C a partial dissociation of the native multimeric form occurred. It is not known if the remaining titre of 50% activity is associated with more heat stable 40-kDa monomers or with partially associated forms of CS-HLLBP.

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

Treatment	HI activity (% control)
1. CS-HLLBP[a] (Control)	100
2. As 1 + Galactosidase (50 U)	100
3. As 1 + Glucosidase (5 U)	100
4. As 1 + Chitinase (0.125 U)	25

[a] The amount of CS-HLLBP was 40 μg in a total assay volume of 100 μl.

The CS-HLLBP native form retained only 50% HI titres below pH 6 but from pH 6 to 10 retained maximum titres. This result indicates that a pH of 6 or greater was required for the formation of the active form, perhaps the multimer, while at pH values closer to its p*I* of 4.7 the ionized states of the protein did not allow for the production of the active form of the CS-HLLBP. This effect may also be related to the thermal effects as discussed earlier.

Treatment of the CS-HLLBP with various glycosidases followed by testing in the HI assay showed that the chitinase enzyme was the only one that resulted in the loss of the binding interaction (Table 2). This is a similar response to that of the RP-HLLBP previously observed and reported (Wititsuwannakul et al., paper #2 in this series). The specific HI titre for CS-HLLBP was 0.23 $\mu g.ml^{-1}$, as determined by the inhibition of HLL induced hemagglutination. This is 6-fold higher than the value of 1.37 $\mu g\ ml^{-1}$, previously reported for RP-HLLBP (Wititsuwannakul et al., paper #2 in this series). Hence, the binding affinity of HLL for the soluble CS-HLLBP was much greater (at least or about 6-fold higher affinity) than that of the bound RP-HLLBP on the small RP. This might explain the colloidal stability of the latex under normal conditions, in that the CS-HLLBP prevents the HLL on the lutoid membrane (if rupture) from interacting with the RP-HLLBP by virtue of its higher affinity and therefore stronger competitive capacity. In addition, a strong and highly specific interaction between HLL and the 40-kDa CS-HLLBP was revealed on SDS–PAGE of the washed pellet obtained after a precipitin reaction (Fig. 4). This precipitin reaction is similar to that commonly employed in an immuno-precipitin assay to demonstrate a highly specific strong interaction, such as antibody/antigen interaction, that produces a precipitate. In this case, the 40-kDa protein reacting specifically with HLL.

2.3. CS-HLLBP as a key factor for preventing latex coagulation

A possible role for CS-HLLBP was further tested using a similar rationale to that used for demonstrating the possibility that RP-HLLBP was the RP ligand that reacted with the HLL to promote aggregation of RP (Wititsuwannakul et al., paper #2 in this series). The assay used was therefore the same as that developed previously to demonstrate RP-HLLBP inhibition of HLL induced RP aggregation. Again preincubation of HLL with

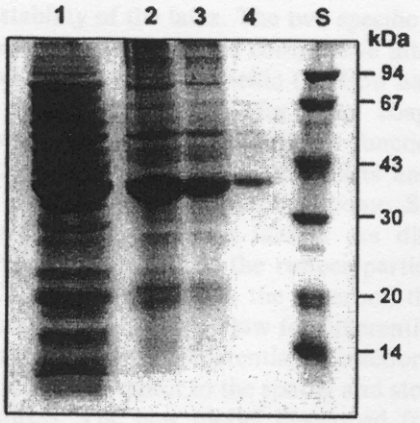

Fig. 4. SDS–PAGE of protein pellet obtained after the precipitin reaction Lane 1: C-serum protein in the 60–80% $(NH_4)_2SO_4$ pellet fraction; lanes 2–4: the serially diluted fraction of washed protein pellet obtained after the precipitin reaction; lane S: standard protein marker.

CS-HLLBP, before being used in the RP aggregation assay, abolished HLL induced RP aggregation in a dose dependent manner (Fig. 5). Under the *in vivo* conditions within the latex vessel, it is hence feasible for the HLL on the lutoid membrane debris to simultaneously link with both the HLLBP in the CS and the HLLBP on the RP via the shared common HLL binding sites. The adsorbed CS-HLLBP, having a higher binding affinity, will prevent direct interactions between the RP-HLLBP and HLL and contribute to the colloidal stability of the rubber particles and prevent latex coagulation. The proposed latex colloidal stability function of CS-HLLBP is similar to that earlier suggested for the C-serum α-globulin (Archer et al., 1963).

2.4. CS-HLLBP levels and their possible uses as rubber yield markers

The very strong inhibition by CS-HLLBP on rubber particles aggregation and hence the latex coagulation as discussed above indicates the possibility that this soluble lectin binding protein could play a key role in prolonging the latex flow time. A possible correlation between the CS-HLLBP level and the latex yield was thus investigated. The accumulated results obtained demonstrated highly significant positive correlation between the CS-HLLBP activity levels (in term of HI titres) for the fresh latex ($r = 0.98$, $P < 0.01$) and the dry rubber yield per tapping ($r = 0.95$, $P < 0.01$) (Fig. 6). These values are significantly higher and more reliable than were our earlier reports on the correlation between latex NAD(P)H quinone reductase [NAD(P)H-QR] activity and rubber yield per tapping (Chareonthipakorn et al., 2002). The NAD(P)H-QR reaction was suggested to support the integrity of the lutoids in the electron transfer process and consequent antioxidant activity for removing free radicals. This activity is very different from that of the CS-HLLBP that contributes to latex stabilization by preventing HLL from interacting with RP-HLLBP through its competitive binding. The CS-HLLBP can play a direct role as an anti-latex coagulating factor by preventing the formation of the rubber coagulum required for plugging of the latex vessels or impeding latex flow. Hence, the CS-HLLBP activity levels are likely to serve as a better marker than that proposed earlier for the NAD(P)H-QR in predicting the yield potential for selected superior rubber producing clones.

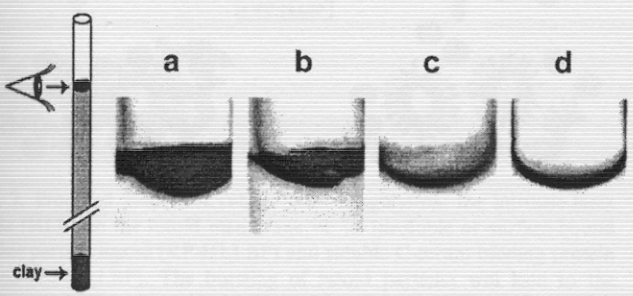

Fig. 5. Inhibitory effect of CS-HLLBP on HLL-induced rubber particle aggregation. The HLL (5 μg) was preincubated with either assay buffer only (a) or buffer solution containing 16.5 (b), 65 (c) or 130 (d) μg of CS-HLLBP before being employed in the rubber particle aggregation assay as described in methods.

2.5. Proposal of a new model for latex coagulation

The physiological functions of HLL in mediating rubber latex coagulation and the sequence of events have been convincingly demonstrated in this series of three related reports. RP-HLLBP bound to the RP surface acts as the ligand for HLL to cause aggregation while the soluble CS-HLLBP acts as an anti-coagulating factor that competes for HLL, prevents aggregation and so maintains

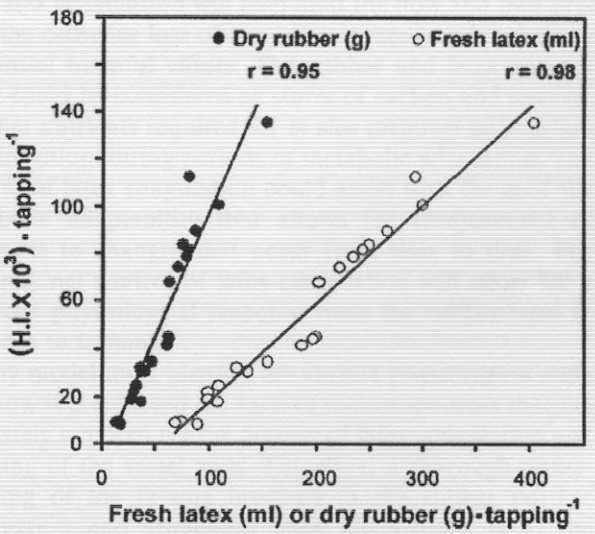

Fig. 6. Correlation between rubber yield and CS-HLLBP activity (HI) per tapping ($n = 22$).

the colloidal stability of the latex. The two specific binding proteins therefore have a dynamic competitive interaction with HLL and their proposed specific function have been incorporated into a new model for latex coagulation (Fig. 7). This model supports the different functions and compartmentalization of the cell constituents and allow for a normal controlled and balanced outcome. Since the coagulating and anti-coagulating factors are differently localized, RP-HLLBP bound to the rubber particles and the soluble CS-HLLBP present in the C-serum, their distinct locales and compartments allow for differential interactions with the HLL. These differential interactions are of a dynamic nature with respect to the spatial and stoichiometric ratio aspects. The new model developed from our finding indicated that the formation of the rubber coagulum, required for latex vessel plugging, is dependent mainly on the interactions between HLL and RP-HLLBP but not those between HLL and CS-HLLBP. For a successful rubber coagulum to form, the number of exposed HLL binding sites should exceed those to be occupied by the CS-HLLBP. Therefore, the higher the number of the remaining exposed HLL binding sites, the better the chance of forming a rubber coagulum.

The mechanism of latex coagulation as proposed in our new model is quite different from the one previously proposed by Gidrol et al. (1994). According to the former model, hevein and chitinase, both localized within the same intra-lutoid compartment, were suggested to play two opposite major roles in the latex coagulation process. The hevein was shown to induce the coagulation of latex by bridging together the rubber particles, in a lectin-like manner, via interacting with the glycosylated 22 kD protein of the rubber particles. Chitinase, on the other hand, was suggested to help prevent latex coagulation by releasing GlcNAc moieties from the 22 kD receptor to block the hevein binding site (Gidrol et al., 1994). The positive effect of chitinase on stabilizing the latex did not fit well with our finding since the HI activities of both coagulating (RP-HLLBP) and anti-coagulating factors (CS-HLLBP) were sensitive to chitinase treatment. However, it may be possible for hevein to play a supportive role by working in parallel with the coagulating factor (RP-HLLBP). This is due to its ability to induce formation of rubber particle aggregates by becoming more accessible to HLL recognition in the rubber coagulum. Moreover, hevein may help to induce formation of a larger rubber coagulum complex by sandwiching between rubber particles attached on different rubber coagulum surfaces. Hence, the partial positive effect of chitinase on latex stabilization may possibly operate through its indirect intervention to diminish the supportive activity of hevein in the formation of the rubber coagulum.

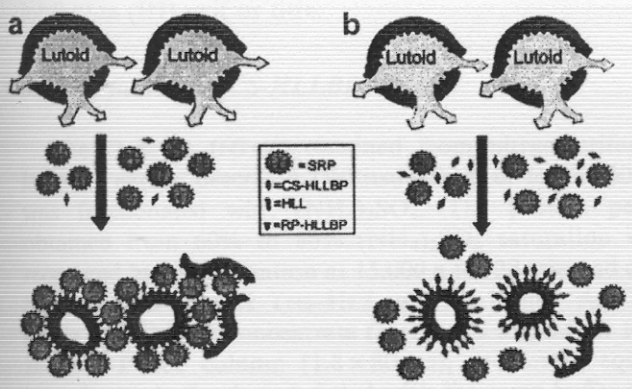

Fig. 7. Proposed model for rubber latex coagulation: the intrinsic latex lectin (HLL) on the lutoid membrane vs. its insoluble or surface-bound rubber particle ligand (RP-HLLBP) and soluble C-serum binding protein ligand (CS-HLLBP). The bursting of lutoid particles will lead to the exposure of HLL that can bind to either RP-HLLBP on the rubber particles, leading to the formation of a rubber coagulum, as depicted in process A or to the soluble CS-HLLBP, forming no coagulum, as depicted in process B. The coagulation of rubber latex will take place whenever process A exceeds B (i.e. at the distal open-end of the latex vessel upon tapping).

Our proposed model agrees well with previously reported parameters involved in latex vessel plugging, that include the important roles of lutoids in latex vessels plugging (Southorn and Edwin, 1968; Southorn and Yip, 1969; Southorn, 1968; Milford et al., 1969; Paardekooper, 1989). Vessel end plugging occurs when the factors that promote rubber coagulum formation out-compete the factors that inhibit coagulum formation. The high correlation between the lutoid bursting index and the intensity or levels of the latex vessel plugging (Yeang and Paranjothy, 1982) has been well documented. The inverse correlation between the lutoid bursting index and rubber yield (Southorn and Yip, 1969) has also been well documented where a higher lutoid bursting index resulted in a sequentially lower yield. Moreover, this proposed intrinsic latex coagulation process, at the tapping or severed ends of the latex vessels, may be one of the primary initial biochemical events that contributes to the complex process of wound healing occurring at the injured tapping site of the bark tissue. The latex coagulation will help limit the flow and thus prevent the excessive loss of stored resources to minimize any sequential harmful effects. The rubber and latex components could be considered as stores of carbon and reserves of other nutrients required for *in vivo* turnover and carbon redistribution during periods of metabolic adaptation. The control of latex coagulation could assist with the need for metabolic adaptability that allows for readjustment of responses to changes that occur from time to time. For example, net control of latex coagulation will allow for a balanced physiological response from the rubber trees in response to tapping.

Any model must identify the correct physiological state and how it is achieved for each of the components that are ultimately required for the process of latex coagulation and plugging of the vessel ends. Therefore, a thorough understanding of the factors involved in the rubber particle aggregation and sequential latex coagulation is of the utmost importance for developing a conceivable mechanism.

2.6. Concluding remarks

The new findings described in this 3 paper series on the identification and characteristics of a latex lectin HLL and two different binding proteins, together with previously reported data, have been used to propose a new model (Fig. 7) that we believe provides a better more logical explanation of the latex coagulation process than does any previous model.

3. Experimental

3.1. Chemicals

DEAE-Sephacel, glycoproteins and chitinase were from Sigma. Bio-gel P-300 from Bio-rad. All other chemicals were of reagent grade.

3.2. Hemagglutination inhibition (HI) assay

The activity of CS-HLLBP was measured by its ability to inhibit hemagglutination induced by HLL. Each CS-HLLBP sample (25 μl) was 2-fold serially diluted with hemagglutination buffer, containing 0.9% NaCl in 50 mM Tris–HCl buffer, in a microtiter U plate. This was followed by addition of HLL solution (25 μl) having a hemagglutination titre of 4 U, the solution then mixed and incubated at room temperature for 20 min before 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 completely inhibited hemagglutination activity induced by HLL was calculated. The inhibition activity was expressed in terms of the hemagglutination inhibition (HI) unit or titre.

3.3. Purification of CS-HLLBP

The C-serum (60 ml) obtained from ultracentrifuged latex as earlier described (Wititsuwannakul et al., 2007) was fractionated with $(NH_4)_2SO_4$. The protein pellet obtained from 60% to 80% $(NH_4)_2SO_4$ saturation was recovered by centrifugation at 15,000 g for 20 min and dialyzed against 50 mM Tris–HCl, pH 7.4 (buffer A). The crude CS-HLLBP concentrate (3 ml) was loaded on to a Bio-Gel P 300 column (1.8 × 80 cm), previously equilibrated with buffer A and eluted with the same buffer at a flow rate of 12 ml/h. Fractions (3 ml) were collected and assayed for HI activity and the peak fractions were pooled, concentrated and further purified on a DEAE-Sephacel column (1.8 × 8 ml), previously equilibrated with 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. The column was then stepwise eluted with buffer A containing 0.1 and 0.2 M NaCl, respectively. The active fractions containing high HI activity were pooled, desalted, concentrated and used for further characterization studies.

3.4. Effect of glycosidases on HI activity of CS-HLLBP

An aliquot containing 50 μl of CS-HLLBP (40 μg) was incubated with 50 μl of glycosidase enzymes (galactosidase, 30 U; glucosidase, 30 U; neuraminidase, 0.15 U and chitinase 0.15 or as indicated) for 30 min at room temperature. After the incubation, the HI activity of CS-HLLBP was determined in each reaction mixture as described in Section 3.2.

3.5. Effect of CS-HLLBP on HLL-induced rubber particle aggregation

A solution (15 μl) containing HLL (5 μg), isolated and purified as earlier described (Wititsuwannakul et al., 2007), was incubated with 15 μl of various amounts, from 16.5 to130 μg of the RP-HLLBP. Incubation without the CS-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 stained, separated and observed as earlier described (Wititsuwannakul et al., 2007).

3.6. Precipitin reaction

The protein pellet from the 60–80% saturated $(NH_4)_2SO_4$ fractionation was dissolved, and dialyzed against buffer A to give a C-serum protein solution (CSP) of 0.52 mg/ml. CSP solution (50 μl), obtained after being serially diluted by 2-fold increments, was added into a precipitin reaction mixture containing 26 μg of HLL in a total volume of 250 μl. After incubation for 1 h at 37 °C, the reaction mixture was kept for 48 h at 4 °C. The precipitate formed was separated by centrifugation (15,000 g, 30 min) and washed with cold PBS. It was then dissolved in 0.5 M NaOH, subjected to SDS–PAGE and visualized after Coomassie Brilliant blue staining (So and Goldstein, 1967).

3.7. Effect of pH and temperature

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

3.8. Polyacrylamide gel isoelectric focusing

Isoelectric focusing was performed on 5% polyacrylamide gel with 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.

3.9. Polyacrylamide gel electrophoresis

SDS-PAGE was performed either in the presence or absence of SDS by the method of Laemmli (1970).

3.10. Protein determination

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

3.11. Correlations between levels of HI activity of CS-HLLBP and the amounts of fresh latex and dry rubber obtained per tapping

Twenty-two 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 separately collected after tapping into an ice-chilled container and the total volume yield per tapping was measured. The amount of dry rubber, obtained from the oven-drying of fresh latex material at 65 °C to constant weight, was used for determination of dry rubber yield per tapping. The fresh latex was fractionated by centrifugation (49,000 g, 4 °C, 45 min) into a top rubber layer, a middle aqueous latex cytosol (C-serum), and a bottom (lutoid) fraction. The C-serum was isolated and directly used for determination of HI activity due to the presence of CS-HLLBP and expressed as total HI activity per tapping. Correlation curves between levels of HI activity of CS-HLLBP and dry rubber and fresh latex yield per tapping were constructed.

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