

Hevein, a Lectin-like Protein from *Hevea brasiliensis* (Rubber Tree) Is Involved in the Coagulation of Latex*

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Xavier Gidrol‡, Hervé Chrestin§, Hui-Lang Tan, and Anil Kush¶

From the Institute of Molecular and Cell Biology, National University of Singapore, 10 Kent Ridge Crescent, Singapore 0511, Republic of Singapore

Hevein, a lectin-like protein is the major protein of vacuolar structures called lutoids in the latex of rubber trees. We have shown both by *in planta* and *ex planta* studies that hevein is involved in the coagulation of latex by bringing together rubber particles. This polyvalent bridging between hevein and rubber particles is mediated by *N*-acetyl-D-glucosamine and involves a receptor glycoprotein of 22 kDa, which is localized on the surface of the rubber particles. The proposed role of hevein helps us to understand the mechanism of coagulation of latex and assigns a physiological intracellular function to one of the smallest lectins.

Most of the natural rubber in the world is obtained from a single plant species, *Hevea brasiliensis* (rubber tree). Natural rubber is obtained from the latex of this plant and is a polymer of isoprene units, in the *cis*-configuration. Latex is the cytoplasm of specialized cells, the laticifers (1). During tapping, laticifers are severed, and the latex flows out of the tree, which upon coagulation and subsequent processing leads to natural rubber. Because latex can be readily obtained in large quantities, it provides an unique opportunity to investigate the biochemical and molecular properties of a single, highly differentiated and specialized cell type. It has been previously shown that the laticifers in fact exhibit differential expression, and several laticifer-specific genes have been isolated and cloned (2, 3).

One of the most predominant proteins in the latex is hevein, which is compartmentalized in lutoids, the vacuolar structures of the laticifer cells. Hevein is a single-chain protein of 43 amino acids, rich in cysteine and glycine (4, 5). Based on the partial amino acid sequence of hevein, a cDNA corresponding to a preproprotein of 20 kDa was cloned. The N-terminal region has homology to wheat germ agglutinin (WGA)¹ and to several chitin-binding proteins, while the C-terminal region is homologous to wound-inducible genes (6).

The coagulation in the rubber tree involves the aggregation of rubber particles and is vital for plant defense against possible pathogen invasion, by blocking the casual wounds or those created during the frequent tapping of trees for latex. This process involves bursting of the lutoid bodies. Based on the results presented here we propose that hevein in latex behaves

like a typical lectin by interacting with a protein of the rubber particle via a sugar linkage and forms aggregates, resulting in the coagulation of latex.

EXPERIMENTAL PROCEDURES

Materials

All chemicals and enzymes, otherwise mentioned, were obtained from Sigma. *N*-acetyl-β-D-glucosaminidase, digoxigenin-labeled WGA, and the glycan differentiation kit were obtained from Boehringer Mannheim. CNBr-activated Sepharose 4B was obtained from Pharmacia LKB Biotechnology Inc. *H. brasiliensis* clone RRIM600 was our source of fresh latex in *ex planta* experiments. Fifteen-year-old trees of clones PB86, PB235, GT1, and WAR4 from the Ivory Coast (Institut de Recherche sur le Caoutchouc en Afrique, experimental plantation) were used in *in Planta* study.

Methods

Ex planta Coagulation of Latex—Lutoids were isolated from latex, and hevein was purified from lutoids serum by high pressure liquid chromatography as previously described (5). The identity of hevein was confirmed using anti-hevein antibodies (kindly provided by Dr. N. Raikhel, Michigan State University) and by partial N-terminal microsequencing of the protein. Chitinase was purified from latex as previously described (7). Freshly tapped latex from 15-year-old RRIM600 trees was diluted 1:100 in 20 mM Tris-HCl, pH 6.8, 0.2 M mannitol, 0.1 mM dithiothreitol, 0.05 mM MgCl₂, 0.1 mM CaCl₂. The final concentration of protein was 0.1 mg/ml⁻¹. One ml of diluted latex was used for each assay directly in a spectrometric cuvette. After 5 min of preincubation at room temperature, 1 μg of purified hevein or bovine serum albumin as control were added to diluted latex. Five min later, hevein-containing samples were further supplemented with other effectors as indicated in the legends of Fig. 1 and Table I. The change in absorbance at 600 nm was monitored as a function of time.

Binding of Labeled GlcNAc to Hevein—The binding of GlcNAc to hevein was monitored by ultrafiltration. Either 800 pmol of purified hevein, 400 pmol of WGA, or 152 pmol of bovine serum albumin were incubated with increasing concentrations of [¹⁴C]GlcNAc (2.1 × 10⁹ Bq mmol⁻¹) in 50 ml of 20 mM Tris-HCl, pH 6.8, 2 μM CaCl₂, 50 μM MgSO₄, 0.1 mM dithiothreitol. The binding of GlcNAc to hevein was also tested in the presence of 1 mM EGTA. After 1 h of incubation at room temperature, aliquots were removed for the count of the total radioactivity, and the rest was ultrafiltered in centricon microconcentrator (amicon) having a molecular mass cutoff of 3000 Da, according to the manufacturer's instruction. Duplicate of the radioactivity in the retentate was counted. The affinity constant was calculated according to Scatchard (8).

Binding of Hevein to *N*-Acetyl-D-glucosamine-Agarose—Binding of hevein to GlcNAc was performed with 10 mg of intralutoid serum, incubated with preequilibrated *N*-acetyl-D-glucosamine-Agarose for 2 h at room temperature, in 20 mM Tris-HCl, pH 6.8, 0.2 mM CaCl₂, 5 mM MgSO₄, 0.1 mM dithiothreitol. The bound fraction could be eluted using 0.1 mM GlcNAc, 1 mM EGTA, or 5 mM EDTA. The binding of hevein to the Agarose-matrix was studied by comparing the protein extracted from the bound Agarose (after boiling in SDS-loading buffer) with the unbound supernatant in the presence or absence of divalent cations as discussed in the figure legends.

Intracellular Location and Purification of the Receptor Protein—The latex was ultracentrifuged (100,000 × *g* for 30 min at 4 °C) and fractionated into bottom fraction (vacuolar structures, so-called lutoids), middle soluble C-serum (mainly cytosol), and the top rubber particles as

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‡ On leave from INRA, 147 Rue de l'Université, 75338 Paris Cedex 07, France.

§ On leave from ORSTOM, BP 5045, 34032 Montpellier Cedex 1, France.

¶ To whom correspondence and reprint requests should be addressed. Tel.: 65-772-6507; Fax: 65-779-1117.

¹ The abbreviation used is: WGA, wheat germ agglutinin.

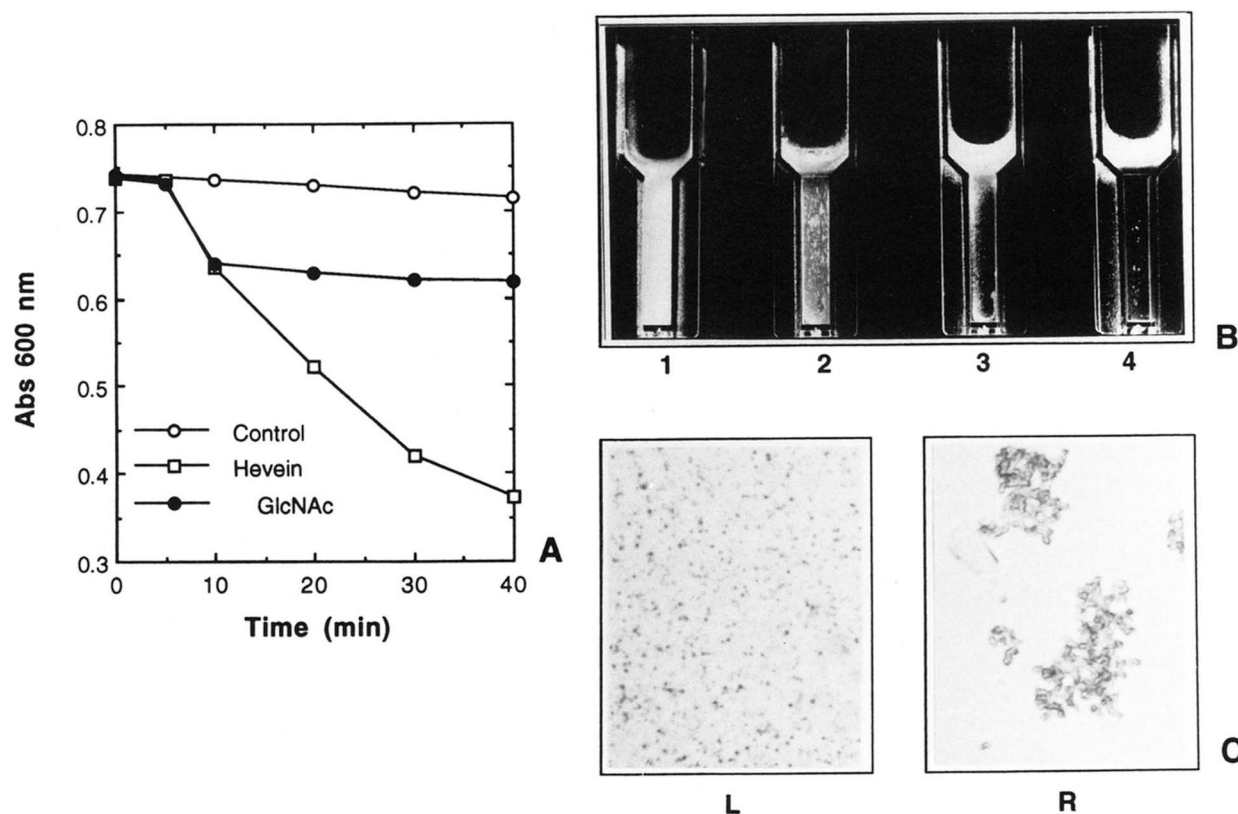


FIG. 1. **Ex planta coagulation of latex.** After 5 min of preincubation, 1 μ g of hevein or bovine serum albumin (*Control*) was added to diluted latex. Five min later, hevein-containing samples were further supplemented with 100 μ M GlcNAc. **A**, the change in absorbance at 600 nm was measured after different time intervals. **B**, the extent of coagulation in the cuvettes could as well be visibly monitored. *Lanes 1, 2, 3, and 4* represent coagulation of latex after 0, 10, 20, and 40 min of addition of hevein, respectively. **C**, coagulation was also observed with a light phase contrast microscope. *Lanes L and R* represent latex before and after 40 min of addition of hevein respectively ($\times 1,200$ -fold magnification).

described previously (9). 50 μ g of total protein from latex, C-serum, and bottom fractions or 10 μ g of rubber particle lysate were prepared as previously described (10) in the presence of 0.1% Triton X-100 and were loaded onto a 12.5% acrylamide SDS-polyacrylamide gel electrophoresis. The Western blot analysis was done using standard techniques (11).

10 μ g of purified hevein was coupled to CNBr-activated Sepharose 4B as per manufacturer's instructions. A rubber particle lysate (100 μ g) was absorbed onto the hevein-coupled column. The bound fraction was eluted using 0.1 M GlcNAc in the same buffer. Fractions of 1 ml were collected and were analyzed by 12.5% acrylamide SDS-polyacrylamide gel electrophoresis. After removal of Triton X-100 by passing the rubber particle lysate through a column of activated charcoal, the lysate was pretreated with *N*-acetyl- β -D-glucosaminidase or chitinases (both purified from latex or from Sigma) for 2 h at 37 $^{\circ}$ C before loading onto the column. The purified 22-kDa protein was then used in the glycan differentiation kit as per manufacturer's instruction.

In planta Experiments—Fresh latex was collected before and after (10 days) treatment with ethephon (12). The clone WAR4 produces about 50 ml of latex per tapping before treatment and responds very well to ethephon, yielding 150–170 ml of latex per tapping. On the other hand, clone GT1 gives 250–300 ml per tapping before treatment but is a poor responder to ethephon stimulation. RNA was extracted from the latex of 10 random trees for each clone, and 20 μ g of RNA was used for each loading, as previously described (2). cDNA of hevein (6) and chitinase (13) were used as probes. The cDNA encoding for β -ATPase (14) was used as an internal loading control (data not shown). Hybridization and subsequent washings were done using standard techniques (11). The coagulation index was defined as the time required to get 50% of the coagulation in *in vitro* conditions as described above, in the absence of any additive.

RESULTS

Hevein Is Involved in Coagulation of Latex—Hevein, which accounts for about 1% of the total latex protein, was purified to homogeneity from luteoid particles. Purified hevein, was added to the diluted latex, and the extent of coagulation was measured by the change in absorbance at 600 nm as a function of

TABLE I
Ex planta coagulation of latex

Effect of various sugars, lectins, antibodies, and enzymes was tested on coagulation of latex. The experiment was performed as described in Fig. 1A. 100 μ M of sugar, 1 μ g of lectin, 5 μ l of 1:1000 diluted antibodies, and 0.1 unit of enzyme were used in the assay. Each value is the mean (of three replicates) decrease in absorbance at 600 nm between 10 and 40 min of incubation at room temperature $\pm$ standard deviation.

Addition	ΔA_{600} (10–40 min)
No addition	0.021 $\pm$ 0.001
Hevein	0.264 $\pm$ 0.007
Bovine serum albumin	0.023 $\pm$ 0.004
Sugars	
Hevein + GlcNAc	0.018 $\pm$ 0.002
Hevein + GalNAc	0.301 $\pm$ 0.015
Hevein + mannose	0.284 $\pm$ 0.010
Hevein + glucose	0.270 $\pm$ 0.010
Hevein + galactose	0.264 $\pm$ 0.012
Hevein + galactosamine	0.290 $\pm$ 0.014
Lectins	
WGA	0.189 $\pm$ 0.003
Concanavalin A	0.038 $\pm$ 0.006
Antibodies	
Hevein + preimmune serum	0.248 $\pm$ 0.010
Hevein + anti-hevein	0.036 $\pm$ 0.003
Enzymes	
Hevein + chitinases ^a	0.046 $\pm$ 0.008
Hevein + <i>N</i> -acetyl- β -D-glucosaminidase	0.040 $\pm$ 0.000

^a Chitinase was either purified from latex or obtained from Sigma.

time (Fig. 1, A and B). The addition of hevein (Fig. 1, A and B) resulted in a sharp decrease in the absorbance due to the aggregation and floating of the rubber particles. This effect could be inhibited by the addition of antibodies specific to hevein but not by preimmune serum (Table I). Among other lectins tested only WGA was found to enhance the rate of coagulation but was

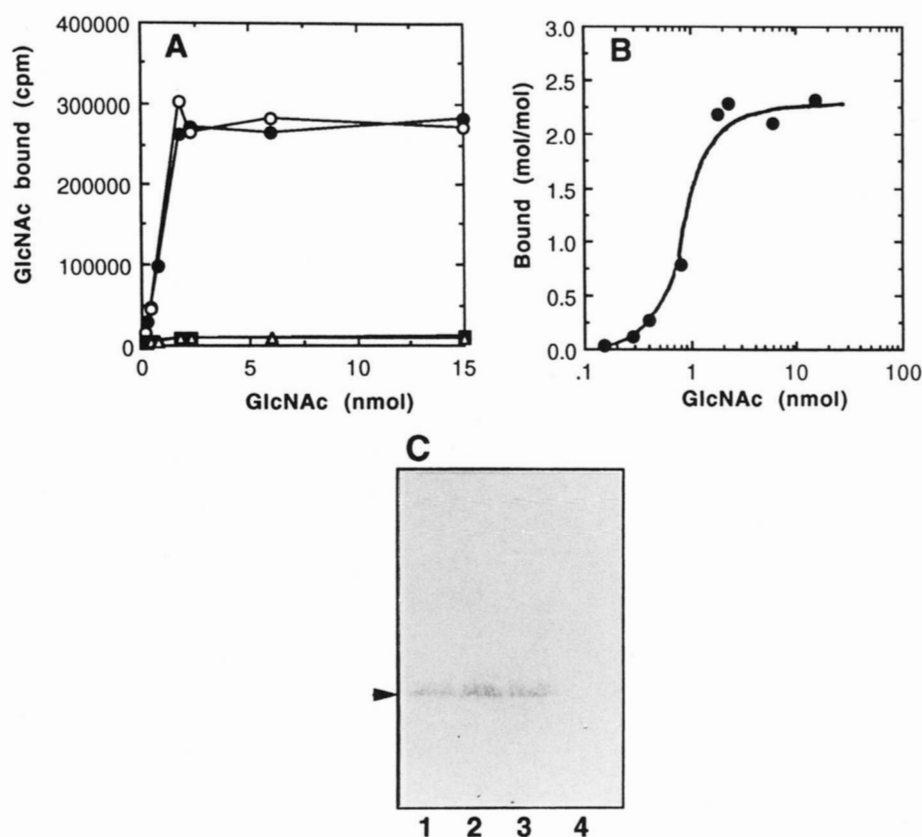


FIG. 2. **GlcNAc binding to hevein.** A, the experiment was carried out as described under "Experimental Procedures." The binding was monitored with 800 pmol of hevein in the absence (●) or presence (△) of 1 mM EGTA, 400 pmol of WGA (○), or 152 pmol of bovine serum albumin (■). Each point is the mean of four independent measurements. The coefficient of variation for each point was in the range of 4–11%. B, the quantity of bound GlcNAc was calculated from data presented in panel A. "Bound" expressed the moles of GlcNAc bound per mole of hevein. C, hevein (black arrow) binds to GlcNAc-agarose (lane 1) in the presence of divalent cations and can be eluted with either GlcNAc (lane 2) or EGTA (lane 3). The binding was found to be inhibited in the presence of EGTA (1 mM) and EDTA (5 mM) (lane 4). Antibodies against hevein were used to detect the protein.

only 72% as efficient as hevein, whereas concanavalin A had no effect (Table I). Expecting sugar specificity for the latex lectin, we studied the effect of various sugars on coagulation. Among them, GlcNAc, which is specific for WGA as well, was found to delay coagulation (Fig. 1A). Other sugars tested had no significant effect under similar conditions (Table I). To further support this, we used two different enzymes, which can remove GlcNAc. *N*-acetyl- β -D-glucosaminidase and chitinases were found to delay coagulation (Table I). The process of coagulation could be monitored microscopically as well, although the results were more difficult to quantify (Fig. 1C).

GlcNAc Binding to Hevein Is Calcium-dependent—The binding of radiolabeled GlcNAc to hevein was monitored to further characterize the interaction between hevein and its specific sugar. The binding of GlcNAc onto 800 pmol of hevein was quantitatively similar to the binding onto 400 pmol of WGA, but unlike WGA the GlcNAc binding onto hevein was Ca^{2+} -dependent, and the addition of EGTA completely inhibited it (Fig. 2A). The semi-log representation of data (Fig. 2B) clearly established the occurrence of two GlcNAc binding sites/monomer of hevein, with an affinity constant of $1.2 \pm 0.7 \times 10^4 \text{ M}^{-1}$, which is consistent with affinity constants of lectin-sugar interaction reported elsewhere (15).

Hevein was then found to bind to GlcNAc-Agarose only in the presence of physiological concentrations of Ca^{2+} (200 μM). In fact, the bound hevein could be eluted with high concentrations of EGTA (1 mM) and EDTA (25 mM) (Fig. 2C). No binding could be detected in the presence of those metal chelators. The binding was shown to be more efficient at pH 5.5 than at pH 7.5.

A 22-kDa Glycosylated Protein Is the Receptor for Hevein on Rubber Particles—Purified hevein was coupled to CNBr-activated Sepharose 4B, and rubber particle lysate was passed through the column. A major protein of 22 kDa was eluted as a single absorbance peak with GlcNAc, but not with other sugars. Pretreatment of rubber particle lysate with either *N*-acetyl- β -D-glucosaminidase or chitinases reduced the quantity of the

protein recovered to 10% as compared with untreated. Antibodies were then raised against the 22-kDa protein, and Western blot analysis demonstrated that antibodies could detect the protein only in the rubber particle lysate, but not in other fractions of latex, neither in the C-serum (middle fraction of latex after ultracentrifugation corresponding to the cytosol) nor in the lutoid lysate (bottom fraction after centrifugation) (Fig. 3A). Immunofluorescence further confirmed the localization of this protein to the surface of the rubber particles (Fig. 3B).

The 22-kDa protein was found to be glycosylated. By using sugar-specific binding of digoxigenin-labeled lectins (*Datura stramonium* agglutinin, *Galanthus nivalis* agglutinin, *Maackia amurensis* agglutinin, peanut agglutinin, *Sambucus nigra* agglutinin, and WGA) we could detect the presence of GlcNAc β 1-4 α and/or GlcNAc β 1-4 α residues as a strong positive signal with labeled WGA (Fig. 4, lane 3). Sugars for other lectins tested, Man α 1-3Man, Man α 1-6Man, Man α 1-2Man, Sia α 2-6Gal, Sia α 2-3Gal, Sia α 2-6GalNAc, Gal β 1-3GalNAc, Gal β 1-4GalNAc, GlcNAc-Ser/Thr, were either not present or not accessible in detectable amounts to the labeled lectin. Treatment of the rubber particle lysate or purified 22-kDa protein with chitinases (data not shown) or *N*-acetyl- β -D-glucosaminidase led to a decrease in relative molecular mass of the protein by approximately 700 daltons, suggesting the removal of about three GlcNAc residues from the protein (Fig. 4, lanes 1 and 2). This was further supported by using digoxigenin-labeled WGA. We could no longer detect the presence of GlcNAc after *N*-acetyl- β -D-glucosaminidase treatment of the 22-kDa protein (Fig. 4, lane 4).

In planta Studies—To correlate our *ex planta* results with the natural behavior of the rubber tree, we studied the expression of both chitinase and hevein genes in various clones of *Hevea* that differed in their rate of coagulation of latex (coagulation index). This rate was found to be responsive to treatment with the hormone ethephon, a generator of ethylene, which is normally used to enhance latex production from rubber trees

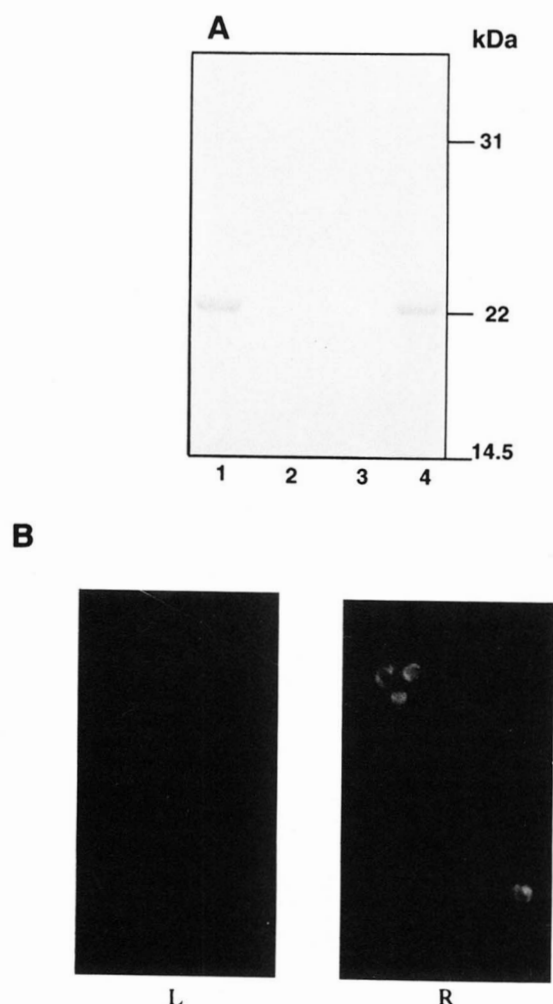


FIG. 3. Interaction between hevein and rubber particles. A, proteins from rubber particle lysate were run in a 12.5% polyacrylamide SDS-polyacrylamide gel electrophoresis and were electrotransferred to a nitrocellulose membrane. The polyclonal antibodies against the purified 22-kDa protein recognize specifically a unique protein in total latex (lane 1) and rubber particle lysate (lane 4) but not in C-serum (lane 2) and bottom fraction (lane 3). B, immunofluorescence analysis of rubber particles was done using either preimmune serum (L) or antibodies (R) (dilution 1:100) against the 22-kDa protein ($\times 3,600$ magnification).

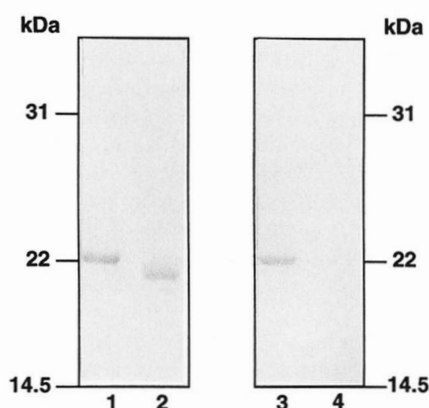


FIG. 4. Glycosylation of the hevein receptor. Treatment of purified 22-kDa protein with *N*-acetyl- β -D-glucosaminidase (0.01 unit/ μ g $^{-1}$ protein) resulted in a decrease in its molecular mass by 700 daltons (lane 2), as determined after staining the gel with Coomassie Blue (lanes 1, no enzymatic treatment). Same treatment also removed the GlcNAc moiety from the protein as determined by digoxigenin labeled WGA (lanes 3 and 4). Lanes 1 and 3 are before and lanes 2 and 4 are after the enzymatic treatment.

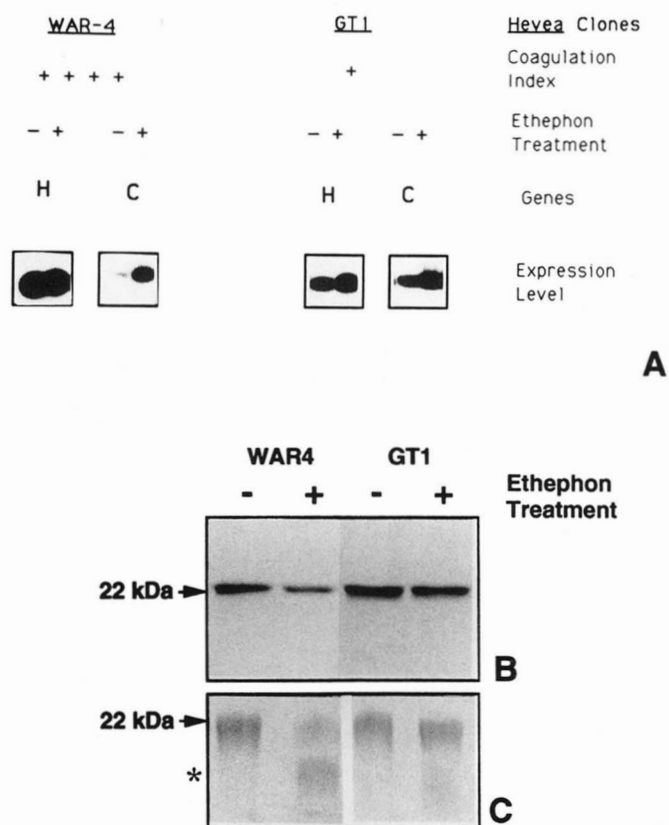


FIG. 5. *In planta* experiments. A, effect of ethephon treatment (- or +) on coagulation of latex from clones GT1 and WAR4. C and H represent, respectively, the level of expression of chitinase and hevein in the latex as determined by Northern blot analysis before (-) and after (+) the treatment with ethephon. GT1 (+) and WAR4 (+++++) differ significantly in their coagulation index. B, effect of ethephon treatment on the level of glycosylation of the 22-kDa protein. As shown in the upper panel, glycan detection was done using digoxigenin-labeled WGA as per manufacturer's instruction, on 50 μ g of rubber particle protein from WAR4 (lanes 1 and 2) and GT1 (lanes 3 and 4) before (lanes 1 and 3) and after 10 days (lanes 2 and 4) of ethephon treatment. C, the lower panel is the same as the upper one, except that instead of labeled WGA, antibodies against 22-kDa protein were used to detect any difference in the amount of this protein in response to ethephon treatment. * indicates deglycosylated protein, which is quantitatively highest in WAR4 in response to treatment. There is no apparent difference in total protein concentration between different genotypes and treatments.

(12), although there exists a wide genetic (clonal) variability in terms of this response. Results from two representative clones, WAR4 and GT1, different in their coagulation index, are presented here. The rubber tree clone WAR4, known to be a poor producer of latex essentially because of its high coagulation index, responded very well to ethephon treatment (3.2-fold increase of latex flow). On the other hand, clone GT1 is a high producer of latex because of a low coagulation index but is a poor responder to treatment. Indeed the latex flow after treatment with ethephon was only increased by 1.3–1.5-fold. It was interesting to note that clone WAR4 had a very high level of constitutive expression of hevein and low chitinase expression before stimulation compared with GT1. Treatment with ethephon increased the chitinase expression in WAR4 to a much higher level (Fig. 5A). This may result in more flow of latex in WAR4, because chitinase inhibits coagulation. Increase in chitinase activity in clone WAR4 resulted in a dramatic decrease in the detection of GlcNAc on the 22-kDa receptor for hevein, indicating a possible role for chitinase in removing the sugar moiety from the receptor protein *in vivo* (Fig. 5B). Western blot analysis confirmed that the 22-kDa protein formed a lower molecular mass species after treatment (Fig. 5B). It fur-

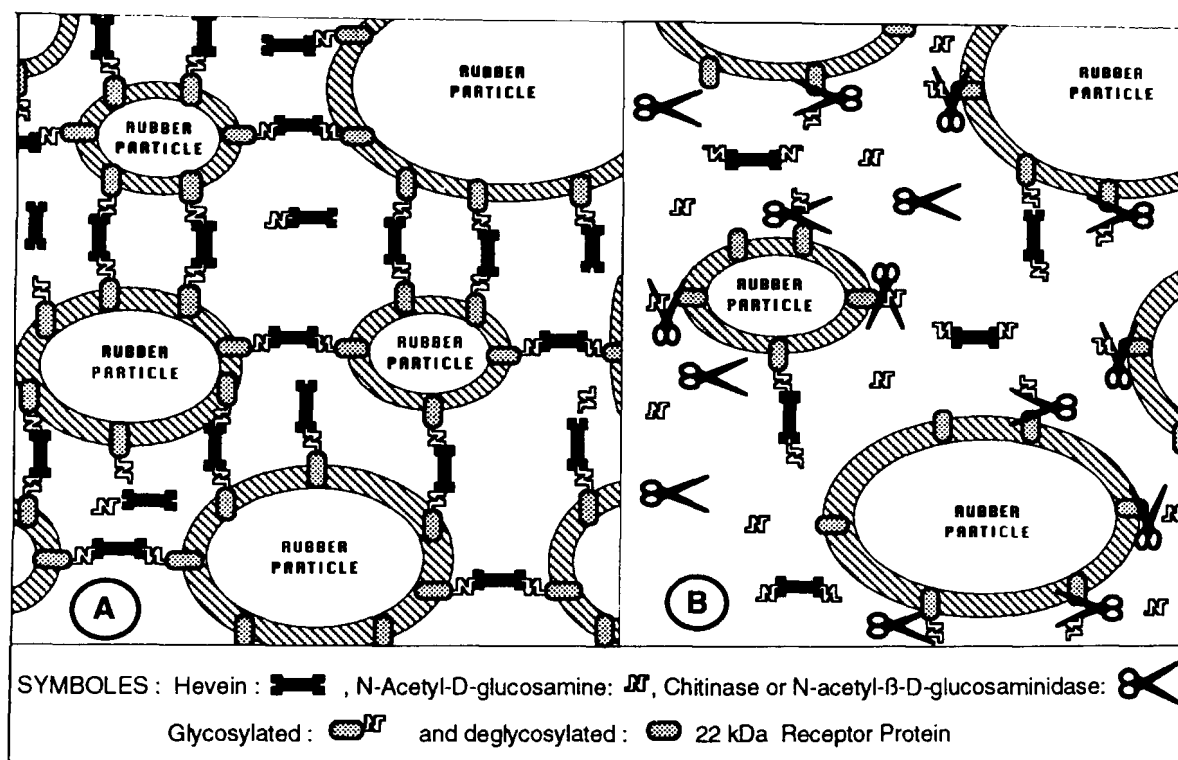


FIG. 6. **Model for coagulation of latex.** A, efficient coagulation of latex occurs at the wounded sites of the laticifers after tapping or *in situ* in case of dry bark disease. Hevein, freed by the bursting of the lutoids bodies, is present in high concentration (together with Ca^{2+}) in the latex cells cytosol. The lectin creates multivalent bridges between rubber particles, through its binding to the GlcNAc moiety of the glycosylated 22-kDa receptor, protein located at the surface of the rubber particles. This stage is associated with low chitinase activity and low level of free GlcNAc in the latex cytosol. Rubber particles are agglutinated, and the latex is coagulated. B, coagulation is inhibited, in normal condition, inside the latex cells. The hevein, mostly compartmentalized in the stable lutoids, is present in low concentration in the latex cytosol. The high chitinase activity provokes the deglycosylation of the 22-kDa receptor protein, increasing in turn the concentration of free GlcNAc, which can saturate the binding sites of hevein still present in the cytosol. Negative charge-charge repulsion keeps the rubber particles in suspension in the cytosol and allows stability of latex.

ther confirmed that the relative amount of deglycosylated protein in clone WAR4 in response to ethephon treatment was much more than in clone GT1 after the same treatment. These observations further supported our *ex planta* data.

DISCUSSION

H. brasiliensis latex is still a unique substance in the plant kingdom. A few hundred milliliters of a single cell type cytoplasm can be obtained from each tree by simply severing the bark. In the present study, we have postulated a possible physiological role for hevein, which was cloned and characterized recently (6). Hevein is found in the lutoid particles (vacuoles) of latex, which, upon tapping, burst open essentially due to the difference in turgor pressure (16). Rubber particles are the site of biosynthesis of natural rubber, which occurs in the cytosol only (17). Under normal conditions hevein does not interact with the rubber particles *in situ* due to its compartmentalization in the lutoids. However in a disease called brown bast or bark dryness, bursting of lutoid particles *in planta* has been reported (18). We think that the bursting of lutoids may allow the rubber particles and hevein to interact, resulting in *in planta* coagulation. Studies using electron microscopy have supported our prediction of *in planta* coagulation in the diseased plants (19). This mechanism of coagulation involving the interaction of hevein with proteins on the rubber particles through a sugar linkage supports the predicted role of hevein as a lectin.

The specific sites at which GlcNAc binds to hevein are not known, but based upon homology between WGA and hevein, one might assume that the binding domain is conserved as already suggested by Wright *et al.* (20). Unlike WGA the bind-

ing GlcNAc onto hevein is Ca^{2+} -dependent. The binding of Ca^{2+} could provoke changes in the conformation of hevein, which in turn are important for the creation or stabilization of the saccharide-binding sites, as it is the case for other metal-dependent lectin such as concanavalin A (15). It might even be possible that Ca^{2+} favor dimerization of hevein *in planta*, allowing in turn binding of GlcNAc. This is in line with the suggestion of Wright *et al.* (20) predicting that hevein should theoretically bind two saccharides provided that dimerization occurs, and a true 2-fold axis of symmetry is formed. Furthermore, the binding of Ca^{2+} likely confers to hevein a high degree of stability protecting it against hydrolysis by proteolytic enzymes present in lutoids. The role of divalent cations and acidic pH in determining the binding affinity of hevein to a specific sugar is further supported by two lines of evidence. Lutoids are known to have an acidic (pH 5.5) and highly buffered content and to be very rich in Ca^{2+} (1.5 mM) as compared with C-serum (0.2 mM) (21). The bursting of lutoids leads to the release of divalent cations along with hevein and provoke the acidification of the immediate vicinity where the process of coagulation starts.

The exact physiological role of lectins is still very obscure although there have been some recent reports of their possible role in protein targeting (22), homing of leukocytes (23, 24), and plant pathogen interactions (25, 26). Evidence from this study helps us to postulate a model explaining the mechanism, at the molecular level, of coagulation of latex. We propose that hevein, a lectin-like protein from the lutoidic vacuoles of *Hevea*, under suitable physiological conditions acts as multivalent bridge in bringing together the rubber particles by interacting with the glycosylated protein of 22 kDa, which is localized on the surface of rubber particles, resulting ultimately in coagulation of latex

(Fig. 6A). The presence of chitinase in the latex inhibits coagulation by removing GlcNAc moiety from the 22-kDa receptor protein on one hand and by increasing the concentration of free GlcNAc in the latex blocking hevein binding sites on the other hand (Fig. 6B). The model we have proposed mostly from our *ex planta* studies have been verified *in planta* as well. We have been able to explain the difference in the coagulation index and duration of latex flow in different genotypes of *Hevea* as such or in response to ethephon treatment. Previous physiological and biochemical studies such as effect of divalent cation and pH on coagulation (27, 28) and latex flow in response to ethephon treatment support the proposed model. Beside the model for latex coagulation and the potential applications in *Hevea* cultivation and rubber technology, our results assign an important intracellular physiological role to a plant lectin, hevein.

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