J. Rubb. Res., **2(2)**, 69–77

Does Hevein Stabilise or Destabilise Rubber Latex?

UKUN M.S. SOEDJANAATMADJA[*,**], TOTO SUBROTO[*,**], JAAP J. BEINTEMA[***,#] AND SOEKENI SOEDIGDO[**,****]

The acidic protein hevein is the major protein in the lutoid-body fraction of rubber latex. The purified protein has a stabilising effect on suspensions of rubber particles in the presence of unfractionated lutoid-body preparations, which contain destabilising basic proteins. The hevein contents in lutoid-body fractions from rubber clones GT 1 and PR 261 are about two times higher than in that of clone LCB 1320, which may explain the faster destabilisation of latex from the latter clone after tapping. Hevein and pseudo-hevein bind chitin and can be isolated by affinity chromatography on a chitin column. Pseudo-hevein binds less strongly to this material than hevein, which can be explained by a replacement of a tryptophan residue by tyrosine in the carbohydrate-binding site of the protein.

The first protein isolated from the lutoid-body fraction of rubber latex was hevein, a small acidic, cystine-rich protein[1] with a polypeptide chain length of 43 residues[2,3]. The three-dimensional structure has been determined by X-ray diffraction[4] and NMR spectroscopy[5]. Hevein is formed from a precursor protein pro-hevein of which the cDNA sequence has been determined[6], as a result of several proteolytic cleavages[7–9]. A minor hevein component, pseudo-hevein, differs at six amino acid positions from hevein[8]. Hevein was found to have affinity for chitin and oligomers of N-acetylglucosamine and inhibits fungal growth[10].

In this paper we show that pseudo-hevein has a lower affinity for chitin than hevein, which can be explained by an amino acid replacement in the chitin-binding site. Gidrol *et al.*[11] reported that hevein is involved in the destabilisation of rubber particles. In a previous paper we have reported that we could not reproduce these results[12]. Here we present evidence that hevein even has a stabilising effect on suspensions of rubber particles and that this feature may explain higher latex yields from rubber clones containing higher hevein contents in their lutoid-body fractions.

MATERIALS AND METHODS

Rubber latex of the clones GT 1, PR 261 and LCB 1320 of *Hevea brasiliensis* was collected at several plantations in Western

* Laboratorium Biokimia, FMIPA, Universitas Padjadjaran, Jl. Singaperbangsa 2, Bandung 40133, Indonesia
** Pascasarjana, Institut Teknologi Bandung, Jl. Ganesa 10, Bandung 40132, Indonesia
*** Biochemisch Laboratorium, Rijksuniversiteit, Nijenborgh 4, 9747 AG Groningen, The Netherlands
**** PAU (Pusat Antar Universitas), Ilmu Hayati, Institut Teknologi Bandung, Indonesia
# Corresponding author

Jaya, Indonesia, cooled in ice and centrifuged at high speed to collect the lutoid-body fraction[13]. This fraction was directly lyophilised and homogenised (Ultra Turrax; Janke & Kunkel, Staufen, Germany) at 4°C in water (4 mg/ml) to which 0.5 g/l sodium thionite was added to inhibit polyphenol oxidases, alternatively, the lutoid-body fraction was submitted to repeated freezing and thawing, followed by centrifigation and lyophilisation of the supernatant (B-serum). Lyophilised B-serum was suspended at 4°C in water to which 0.5 g/l sodium thionite was added. After centrifugation, solutions were 100% saturated with ammonium sulphate and centrifuged again. Precipitates were dissolved in small volumes of 0.2 *M* acetic acid and submitted to gel-filtration on a column of *Sephadex G-25*. Second peaks contained hevein and initial peaks, other proteins. The hevein containing peaks were lyophilised and submitted to reverse-phase HPLC on a Nucleosil 10 C18 (30 cm × 0.45 cm) with a gradient of 0%–70% acetonitrile in 0.1% trifluoroacetic acid for 60 min at a flow rate of 1 ml/min. The eluent was monitored at 214 nm and the peaks collected manually. Polyacrylamide gel electrophoresis of protein mixtures was performed on 15% gel in sodium dodecylsulphate[14].

C-serum of rubber latex and rubber particles (clone PR 255) were isolated as described by Moir[13]. The stabilising effects of lyophilised C-serum preparations and hevein on rubber particle suspensions in the presence of destabilising quantities of lyophilised B-serum were estimated as described by Yeang[15]. Chitin was prepared by acetylation of chitosan as described by Molano *et al.*[16] 1 mg quantities of hevein and pseudo-hevein were submitted to affinity chromatography on a chitin column (9 cm × 1.4 cm) in 50 mM sodium acetate, pH 3.8[10], with elution of the bound hevein and pseudo-hevein with 1 *M* acetic acid.

RESULTS AND DISCUSSION

B-sera obtained from 1 litre of rubber latex were lyophilised, suspended in water containing 0.5 g/l sodium dithionite and 100% saturated with ammonium sulphate. The precipitates were dissolved in 0.2 *M* acetic acid and subjected to gel filtration on a column of *Sephadex G-25*. *Figures 1* and *2* show the absorbancy patterns at 280 nm and 260 nm obtained with B sera from rubber clones PR 261 and LCB 1320, respectively. The pattern obtained with B-serum from clone GT 1 is similar to that obtained from clone PR 261[9]. The second protein peak contains only hevein (and pseudo-hevein), while all other proteins are present in the first one. Weights of freeze dried B-sera, and of the two peaks, after freeze drying, are summarised in *Table 1*, together with some other characteristics of the collected latices.

Less protein is present in B-serum of latex of clone LCB 1320, than in those of the other two clones. The lower protein content in the first peak can be accounted for by the absence of one of the two hevamine (chitinase) components and the much lower glucanase content[12]. However, most striking is the low hevein content in this clone; less than 60% of those in the other two clones, resulting also in a much reduced ratio of the two protein peaks in *Figure 2*, compared to *Figure 1*. The lower hevein content in B-serum of clone LCB 1320 compared to those in other clones is also evident from an electropherogram of B-serum proteins separated by polyacryl amide gel electrophoresis in sodium dodecyl sulphate, although the diffused bands of hevein in all four samples suggest lower quantities than the actual contents (*Figure 3*).

Isolations of lutoid-body proteins have also been performed starting not with lyophilised B-sera, but with preparations obtained by homogenisation of freeze dried lutoid-body

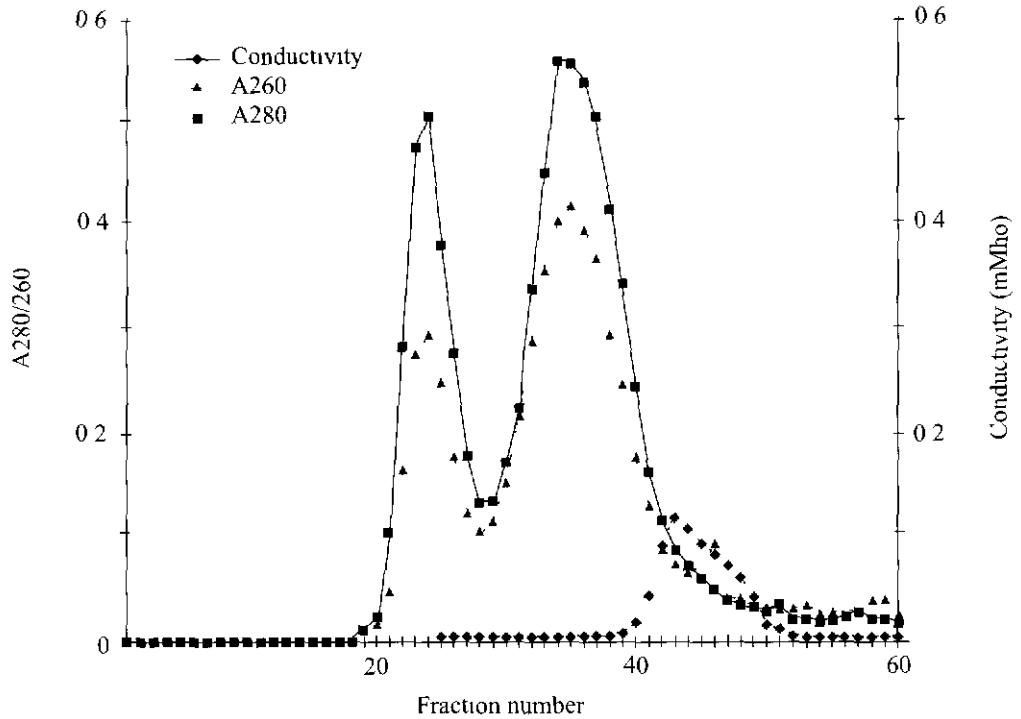

Figure 1 Gel-filtration of a 100% saturated ammonium sulphate precipitate of 100 mg lyophilised B serum of rubber clone PR 261 on a Sephadex G-25 *column (3×50 cm) Elution with 0 2* M *acetic acid Fractions of 5 0 ml were collected*

TABLE 1 PROTEIN CONTENTS OF B-SERA AND OTHER CHARACTERISTICS OF LATEX OF RUBBER TREE CLONES

Rubber tree clone	GT 1	PR 261	LCB 1320
Latex yield per tree, per day	550 – 700 ml	650 – 700 ml	150 – 200 ml
B-serum from 1 l latex	150 – 170 ml	140 – 150 ml	100 – 125 ml
Weight B-serum (after freeze drying)	9 3 g	9 2 g	9 1 g
Protein content of B-serum fractions			
Sephadex G-25 peak I	7 6 mg	7 2 mg	5 6 mg
Sephadex G-25 peak II (hevein)	8 8 mg	8 9 mg	5 1 mg
Ratio peak II peak I	1 2	1 2	0 9

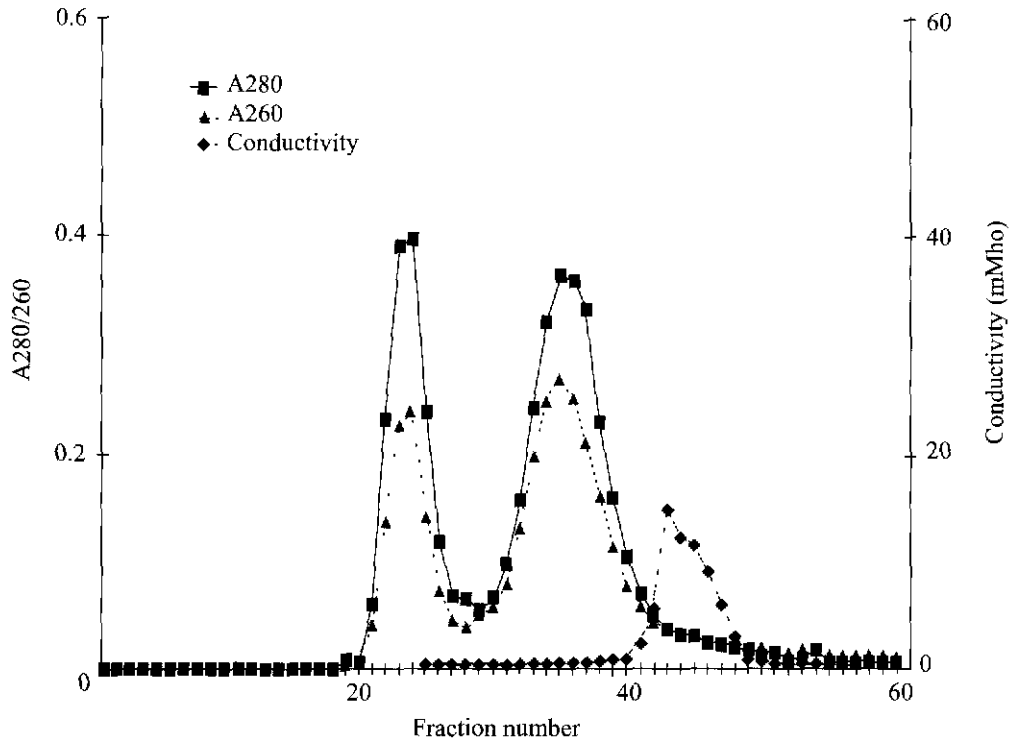

Figure 2 Gel-filtration of a 100% saturated ammonium sulphate precipitate of 100 mg lyophilised B-serum of rubber clone LCB 1320 on a Sephadex G-25 *column.*

fractions. Generally no differences were observed in properties or quantities of the purified protein. However, when HPLC was performed on the second *Sephadex G-25* peak for the separation of pseudo-hevein from hevein, more of a third peak was obtained from preparations obtained by homogenisation of freeze dried lutoid-body fractions than from B-serum (*Figure 4*). This third peak represents modified hevein, in which the N-terminal glutamate has been cyclised to a pyroglutamate residue[8]. Evidently the procedure to obtain B-serum by repeated freezing and thawing of the lutoid-body fraction, is milder than homogenisation, during which rather high local energy expenditures may occur.

Basic proteins destabilise rubber particle suspensions, and this may be one of the reasons that rupture of lutoid-body membranes during rubber tapping causes coagulation of the latex[17]. In a previous paper we have presented evidence that purified preparations of hevamine and glucanase destabilise suspensions of rubber latex particles, with a relatively much stronger destabilising capacity of the latter protein[12].

Yeang[15] has described that the cytoplasm fraction (C-serum) of rubber latex has a stabilising effect on a suspension of rubber particles. We have repeated these experiments by estimation of a creaming effect on a suspension of rubber particles by the addition of

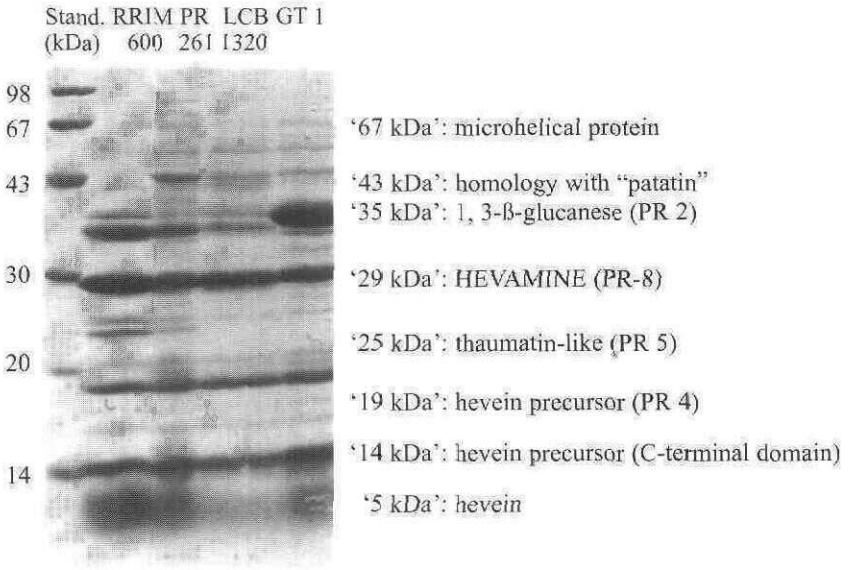

Figure 3. Polyacrylamide gel electrophoresis in sodium dodecylsulphate on a 15% gel of ca *50 µg quantities of lutoid-body fractions of clones GT 1, PR 261, LCB 1320 and RRIM 600 (gift from Dr. H.Y. Yeang, Rubber research Institute of Malaysia, Kuala Lumpur, Malaysia). The left/right lane indicates standard proteins with known* M_r*s in kDa.*

B-serum, and the protection exhibited by increasing amounts of C-serum (*Table 2*). In addition to B- and C-serum we have also added hevein to the suspension. Hevein is an acidic protein. It has been claimed that this protein also destabilises suspension of rubber particles because being a lectin it may bind glycoproteins surrounding rubber particles[11]. However, rubber particles are part of the cytoplasm[18], which is not the normal location of glycoproteins[19]. Therefore it is not surprising that we could not reproduce the destabilising effect of hevein[12]. Our current observations indicate that the acidic hevein even stabilises suspensions of rubber particles. We could confirm the observations by Yeang[5] that increasing quantities of lyophilised C-serum have a stabilising effect, as indicated by scoring creaming (*Table 2*) and flocculation (not shown) of rubber particle suspensions. As shown in *Table 2* the presence of 0.5% hevein in the mixtures decreased creaming. Although a longer incubation time (10 h) increased the creaming score, compared with that after 1 h, the stabilising effect of hevein was still evident from the data presented in *Table 2*. However, the presence of hevein had less influence on the flocculation properties of the mixtures (not shown).

Rubber latices from clones GT 1 and PR 261 coagulate slower than that of clone LCB 1320, although they contain higher quantities of the positively charged destabilising proteins hevamine and glucanase. In a previous paper[12] we have suggested that a high glucanase content in latex may cause more degradation of callus tissue and therefore a longer lasting latex flow. However, the hevein content may be a more predominant factor and if this protein has a stabilising effect on suspensions of rubber

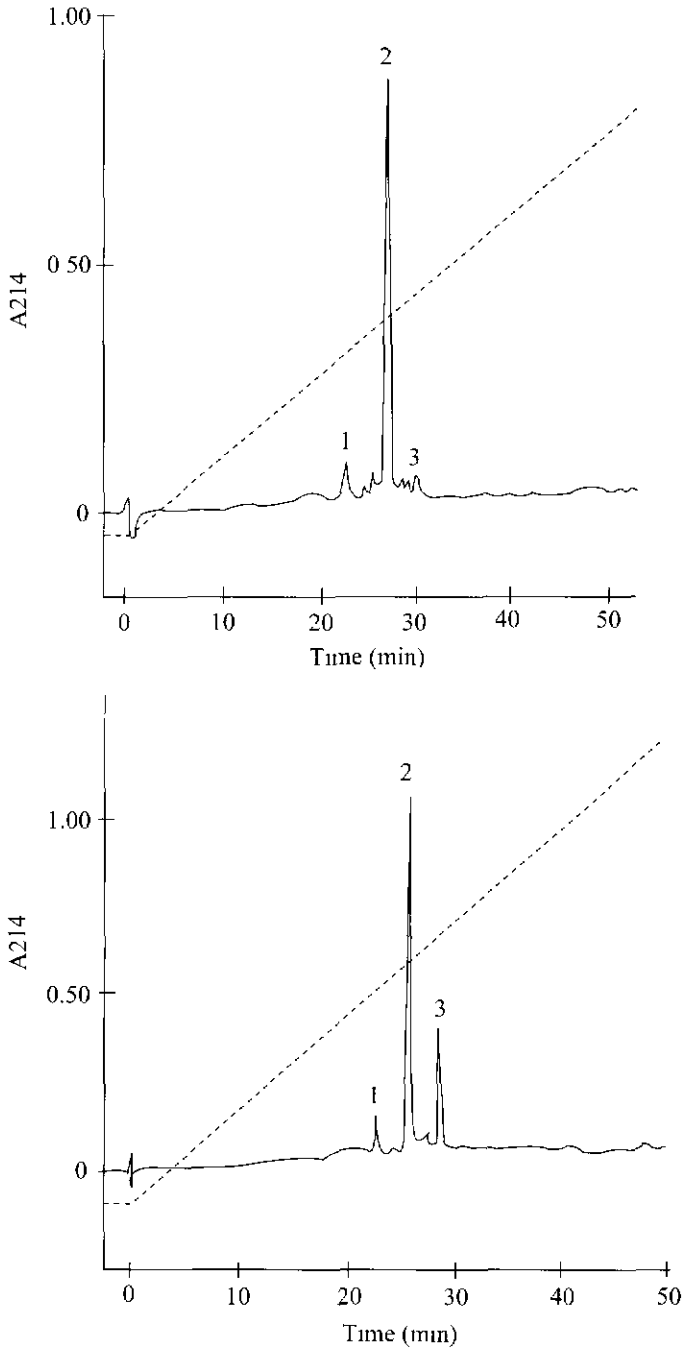

Figure 4 Reverse-phase HPLC separation of hevein components.
Peak 1: pseudo-hevein; Peak 2 hevein. Peak 3: second minor component
Top preparation from B-serum, Below preparation from freeze-dried bottom fraction

TABLE 2. STABILISING EFFECT OF C-SERUM AND HEVEIN PREPARATIONS ON RUBBER PARTICLE SUSPENSIONS

C-serum	No hevein		0.5%	
	Yeang[15]	Current research	Current research	
	15 min	1 h	1 h	10 h
1%	1.8	1.8	0.8	1.2
2%	ND	1.6	0.3	0 8
5%	1.4	1.5	0.0	0.6
10%	1.0	1.1	0.0	0.4
20%	0.5	1 0	0 0	0 2
50%	0.0	0.0	ND	ND

Test suspensions contained 1% rubber particles (w/v), 5% freeze dried B-serum (w/v) and indicated quantities of freeze-dried C-serum (w/v) and hevein (w/v) in water. Creaming of 'zone 1' rubber particle suspensions after 1 h or 10 h was measured as described by Yeang[15], with the results expressed on a scale from 0.0 (no creaming) to 2.0 (maximum extent of creaming). Results are the mean of four experiments ND, not determined

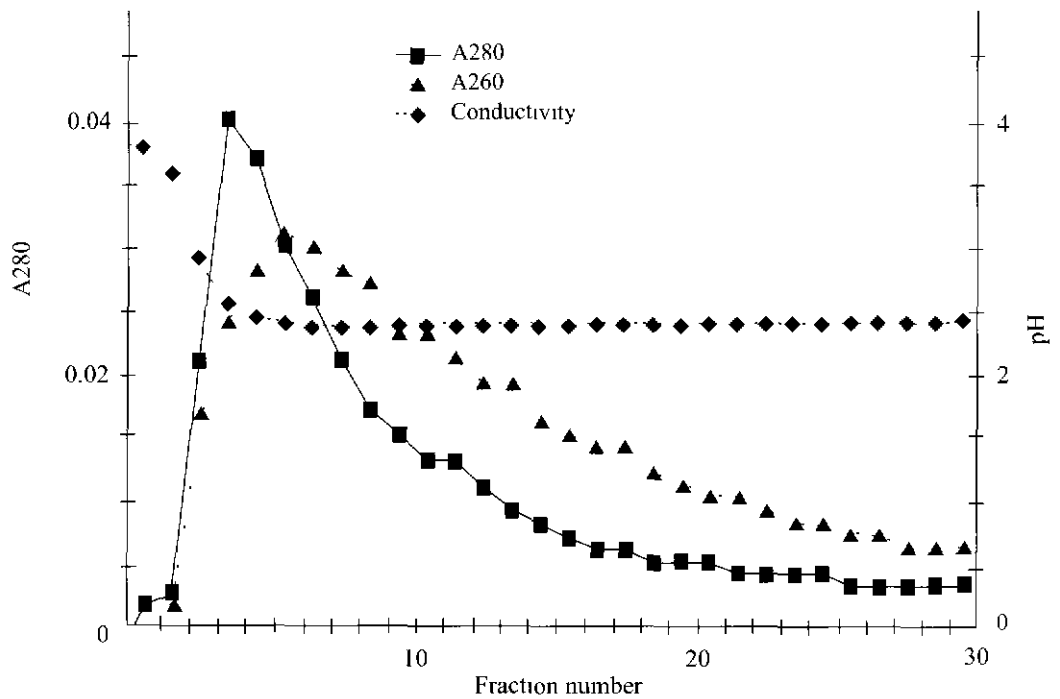

Figure 5 Affinity chromatography of hevein Proteins were eluted with 1 M *acetic acid*

particles, its much lower content in latex of clone LCB 1320 (*Table 1*) may result in faster coagulation

Probably antifungal properties of hevein are more relevant Van Parijs *et al*[9] have demonstrated its chitin-binding antifungal properties Chitin and N-acetylglucosamine binding properties of homologous domains in other proteins have been demonstrated by crystallographic[20] and quantitative binding studies[21] The glucosamine binding site of hevein has been investigated by ^{1}H-NMR spectroscopy[22] and by CIDNP nuclear magnetic resonance studies[23] Crude preparations of hevein contain a small amount of a pseudo-hevein, a variant of which it is not known if it has any physiological function An interesting difference between pseudo-hevein and hevein is the replacement of a tryptophan residue by a tyrosine at position 21 of the sequence in the carbohydrate binding site We have investigated the influence of this replacement by investigation of the elution properties of hevein and pseudo-hevein during affinity chromatography on a chitin column *Figure 5* shows indeed that pseudo-hevein elutes earlier than hevein, in agreement with a lower binding strength to this polysaccharide

ACKNOWLEDGEMENTS

These studies were supported by the Directorate of Research Development and Community Services (Project 'Hibah Bersaing'), Directorate of higher Education, Department of Education and Culture (Indonesia) and the Netherlands Foundation for the Advancement of Tropical Research (WOTRO) We thank Djuharia S for help in collecting rubber latex and preparation of lutoid-body fractions

Date of receipt September 1998
Date of acceptance June 1999

REFERENCES

1 ARCHER, B L (1960) The Proteins of *Hevea brasiliensis* Latex Isolation and Characterization of Crystalline Hevein *Biochem J* **75**, 236

2 WALUJONO, K , SCHOLMA, R A , BEINTEMA, J J , MARIONO, A AND HAHN, A M (1975) Amino Acid Sequence of Hevein *Proc Int Rubber Conf, Kuala Lumpur*, **2**, 518–531

3 BEINTEMA, J J AND PELUMANS, W J (1992) The Primary Structure of Stinging Nettle (*Urtica dioica*) Agglutinin, a Two-domain Member of the Hevein Family *FEBS Lett* **299**, 131

4 RODRIGUEZ-ROMERO, A , RAVICHANDRAN K G AND SORIANO-GARCÍA, M (1991) Crystal Structure of Hevein at 2 8 Å Resolution *FEBS Lett* , **291**, 307

5 ANDERSEN, N H , CAO, B , RODRIGUEZ-ROMERO A , AND ARREGUIN, B (1993) Hevein NMR Assignment and Assessment of Solution-state Folding for the Agglutinin-toxin Motif *Biochemistry*, **32**, 1407

6 BROEKAERT W , LEE, H I , KUSH, A , CHUA, N H AND RAIKHEL, N V (1990) Wound-induced Accumulation of mRNA Containing a Hevein Sequence in Laticifers of Rubber Tree (*Hevea brasiliensis*) *Proc Natl Acad Sci* , **87**, 7633

7 LEE, H I BROEKAERT, W AND RAIKHEL, N V (1991) Co- and Post-translational Processing of the Hevein Preproprotein of Latex of the Rubber Tree (*Hevea brasiliensis*) *J Biol Chem* , **266**, 15944

8 SOEDJANAATMADJA, U M S , HOFSTEENGE, J , JERONIMUS-STRATINGH, C M , BRUINS, A P AND BEINTEMA, J J (1994) Demonstration by Mass Spectrometry that Pseudo-Hevein and Hevein have

Ragged C-terminal Sequences *Biochim Biophya Acta*, **1209**, 144

9 SOEDJANAATMADJA, U M S , SUBROTO, T AND BEINTEMA, J J (1995) Processed Products of the Hevein Precursor in the Latex of the Rubber Tree (*Hevea Brasiliensis*) *FEBS Lett* , **363**, 211

10 VAN PARIJS, J , BROEKAERT, W F , GOLDSTEIN, I J AND PEUMANS, W J (1991) Hevein An Antifungal Protein from Rubber Tree (*Hevea brasiliensis*) *Latex Planta*, **183**, 258

11 GIDROL, X , CHRESTIN, H , TAN, H L AND KUSH, A (1994) Hevein, a Lectin-like Protein from *Hevea brasiliensis* (Rubber Tree) is involved in the Coagulation of Latex *J Biol Chem* , **269**, 9278

12 SUBROTO, T , VAN KONINGSVELD, G , SCHREUDER, H A , SOEDJANAATMADJA, U M S AND BEINTEMA, J J (1996) Chitinase and β-1, 3-glucanase in the Lutoid-body Fraction of *Hevea* Latex *Phytochemistry*, **43**, 29

13 MOIR, G F J (1959) Ultracentrifugation and Staining of *Hevea* Latex *Nature (London)*, **184 (4699)** , 1926

14 LAEMMLI, U K (1970) Cleavage and Structural Proteins during Assembly of Head of Bacteriophage T-4 *Nature (London)* **227**, 680

15 YEANG, H Y (1989) Characterisation of Rubber Particle Destabilisation by B-serum and Bark Sap of *Hevea brasiliensis J nat Rubb Res* , **4(1)**, 47–55

16 MOLANO, J , DURAN, A AND CABIB, E (1997) A Rapid and Sensitive Assay for Chitinase Using Tritiated Chitin *Anal Biochem* , **83**, 648

17 SOUTHORN, W A AND YIP, E (1968) Latex Flow Studies III Electrostatic Considerations in Colloidal Stability of Fresh Latex from *Hevea brasiliensis J Rubb Res Inst alaya*, **20(4)**, 201–215

18 RUINEN, J (1950) Microscopy of the Lutoids in *Hevea* Latex *Ann Bogoriensis*, **1**, 27

19 STRYER L (1955) Biochemistry, 4th Edition, Chapter 35

20 WRIGHT, H T , SANDRASEGARAM, G AND WRIGHT, C S (1991) Evolution of a Family of N-acetylglucosamine Binding Proteins Containing the Disulfide-rich Domain of Wheat Germ Agglutinin *J Mol Evol* , **33**, 283

21 SHIBUYA, N , GOLDSTEIN, I J , SHAPER, J A , PEUMANS, W J AND BROEKAERT, W F (1986) Carbohydrate Binding Properties of the Stinging Nettle (Urtica dioica) Rhizome Lectin *Arch Biochem Biophys* , **249**, 215

22 ASENSIO, J L , CAÑADA, F J BRUIX, M , RODRIGUEZ-ROMERO, A AND JIMINEZ-BARBERO, J (1995) The Interaction of Hevein with N-acetylglucosamine-containing Oligosaccharides Solution Structure of Hevein Complexed to Chitobiose *Eur J Biochem* , **230**, 621

23 SIEBERT, H -C , VON DER LIETH, C -W , KAPTEIN, R BEINTEMA, J J, DIJKSTRA, K , VAN NULAND, N , SOEDJANAATMADJA, U M S , RICE, A , VLIEGENTHART, J F G , WRIGHT, C S AND GABIUS H -J (1997) Role of Aromatic Amino Acids in Carbohydrate Binding of Plant Lectins Laser Photo Chemically Induced Dynamic Nuclear Polarization Study of Hevein-domain Containing Lectins *Proteins*, **28**, 268