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# *Isolation of a Protease Active at Neutral pH with a Molecular Mass of 65 kDa from the Lutoid-body Fraction of* Hevea brasiliensis *Latex*

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*A protease with a molecular mass of 65 kDa was isolated from the lutoid-body (vacuolar) fraction of latex of the rubber tree,* Hevea brasiliensis, *and assigned to one of the approximately ten protein bands visible on SDS-PAGE patterns of this fraction. The enzyme had an optimum activity at pH 7.4 and was inhibited at this and higher pH values in the cytosol fraction of rubber latex. There might be a small proteolytic activity at the low internal pH of lutoid-bodies. The 19 kDa precursor of hevein disappeared during incubation of lutoid-body preparations at neutral pH, but not at lower pH. A possible general role of the 65 kDa protease in processing of vacuolar proteins needs more investigation*

**Key words**: *Hevea brasiliensis*; rubber latex; fraction; lutoid bodies; vacuoles; protease; protein; isolation; hevein; SDS-PAGE

Latex of the rubber tree, *Hevea brasiliensis*, is the cytoplasm of specialised cells known as laticifers. About 15% of its volume consists of lutoids, which are organelles of vacuolar origin containing a limited number of major proteins. Upon centrifugation, rubber latex separates into a layer of rubber particles, the cytosol and lutoid-body fraction[1]. About half of the protein content of the lutoid-body fraction can be accounted for by hevein, a small protein with antifungal properties[2], which is formed by proteolytic processing of a precursor protein, of which the cDNA sequence is known[3]. Initial processing of the precursor involves removal of the N-terminal signal peptide[4] and of a vacuolar targeting sequence[5]. Hevein is formed from the processed precursor (19 kDa) by cleavage between this domain (5 kDa) and a C-terminally located one (14 kDa), followed by further removal of residue at the C-terminus of hevein resulting in a ragged structure[6]. The processed precursor (19 kDa) and the C-terminal domain of 14 kDa are also present in the lutoid-body fraction, but at molar ratios of about 1:30 relative to hevein[5].

In order to collect more information about proteolytic steps in the processing of the precursor of hevein, we started an investigation on the presence of proteolytic activity in the

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lutoid-body fraction of rubber latex Here we describe the isolation of a protein with a molecular mass of about 65 kDa, which accounts for this activity at neutral pH The hevein precursor of 19 kDa is stable at the low intrinsic pH value of a lutoid-body preparation, but disappears during incubation at higher values The 65 kDa protein may be involved in processing of nascent proteins at neutral pH before they reach the more acidic vacuolar environment

## MATERIALS AND METHODS

### Isolation of the Protease

Rubber latex of clone GT1 of *Hevea brasiliensis* was collected in the plantations of Gunung Hejo Panglejar-Purwakarta (PTP Nusantara VIII, Western Java, Indonesia) It was then cooled in ice and centrifuged at high speed to enable the collection of the lutoid-body fraction[1] This fraction was submitted to repeated freezing and thawing, followed by centrifugation and lyophilisation of the supernatant (B-serum)[7] Lyophilised B-serum was suspended at 4°C in a small volume of 10 mM sodium acetate buffer pH 6 0 or 0 2 M acetic acid, and after centrifugation submitted to gel-filtration on a column (50 cm × 3 cm) of Sephadex G-25 at room temperature, which showed a first peak with all proteins, except for hevein, which eluted as second peak The first peak from the gel filtration at pH 6 0 was dialysed overnight against 0 04 M sodium borate buffer, pH 8 9, centrifuged and submitted to cation-exchange chromatography on a carboxymethyl-cellulose column CM32, which separates unretarded acidic proteins from the major basic proteins hevamine and β-1,3-glucanase The peak with unretarded proteins was lyophilised, dialysed against 0 1 M sodium phosphate buffer, pH 7 6, and subjected to gel filtration on a column (40 cm × 1 6 cm) of Ultragel AcA34 The first peak, that contained proteolytic activity, was lyophilised, dialysed against 0 05 M Tris-HCl buffer, pH 8 0, and submitted to anion exchange chromatography on a DEAE-cellulose column DE52 (30 × 1 5 cm), with a gradient of 0–0 1 M NaCl The protein was eluted into two peaks, of which the first one exhibited proteolytic activity

### Analytical Methods

SDS-PAGE of protein preparations was performed on 15% gels[8] Proteins on all gels were stained with Coomassie Brilliant Blue Protease determinations were performed as described by Reimerdes and Klostermeyer[9] Protein samples (50 µL–125 µL) in 0 1 M sodium phosphate buffer, pH 6 6, were mixed with 0 5 mL substrate (20 mg N, N'-dimethylated casein, Sigma) in the same buffer and incubated for 10 min at 37°C After incubation, 0 5 mL 8% (v/v) trichloroacetic acid was added, followed by centrifugation and measurement of the absorbance of the supernatant at 280 nm Enzyme activities are presented as arbitrary units The pH optimum of the enzymic activity was determined as above with 50 µL pure enzyme (20 µg mL$^{-1}$ in water) and measurement of the activity from pH 6 6 – pH 8 0 in 0 1 M sodium phosphate buffer

The type of reaction catalysed by the protease in the latex of *Hevea brasiliensis* was investigated by using several inhibitors (purchased from Sigma) according to the protocol (*Table IV*) presented by Barrett[10]

The cytosol fraction of rubber latex contains a protease inhibitor[11] A preparation containing this inhibitor was obtained from the cytosol fraction (C-serum) by centrifugation of rubber latex at high speed[1] Proteins in the C-serum were precipitated by saturation with ammonium

sulphate, dissolved in water, dialysed against water and lyophilised The ability of 50 mg quantities of this preparation to inhibit the isolated protease was investigated by measurement of the activity of 1 µg of this enzyme in the pH range of 6 6 – 8 0, as described above

**Protein Processing**

The stability of the 19 kDa hevein precursor was investigated by observing the intensity of bands stained with Coomassie Brilliant Blue of samples containing this protein after the initial gel filtrations of lutoid-body fractions on Sephadex G-25 in 0 2 M acetic acid and in 0 2 M sodium acetate buffer, pH 6 0, at room temperature (about 25°C), and after incubation of dialysed and lyophilised samples of the first peak from the gel filtration at pH 6 0, dissolved (4 mg mL $^{-1}$) in 200 mM Tris-HCl buffer, pH 7 0, at 37°C for 1 h–8 h

## RESULTS AND DISCUSSION

The lutoid-body fraction of rubber latex contains a small number of major proteins, and several less predominant ones Most of these have been structurally and functionally characterised[12,13] Here it was shown that the SDS-PAGE band of the largest of the less predominant proteins with an apparent molecular mass of 65 kDa could be assigned to a protease This enzyme was isolated by several gel filtrations and chromatographies on ion exchangers At each step, only one peak with protease activity could be demonstrated Gel filtration on Ultragel AcA34 of the acidic proteins, not bound by CM-cellulose at high pH, showed that the protease was present in the first peak, eluting before a peak containing predominantly a protein with a mass of 55 kDa[14], and a larger peak with other acidic proteins (*Figure 1*) SDS-PAGE patterns of the peaks are shown in *Figure 2* Final purification of the protease was obtained by anion exchange chromatography, on which it was separated from residual quantities of a 43 kDa protein (*Figure 3*), which is a homologue of patatin[1] that may form dimers[15], explaining its behaviour on the Ultragel AcA34 column

On SDS-PAGE, only one protein band was visible with a molecular mass of about 65 kDa (*Figure 4*) About 1 5 mg pure protein was obtained from per gram of lyophilised lutoid-body fraction A purification of about 40-fold was obtained with a recovery of 25% of the initial enzymic activity

*Figure 5* shows that the enzyme has an optimum at neutral pH It may have little activity at the internal pH of lutoid bodies, which is about 5 5 and 1 5 unit lower than that of the surrounding cytosol[16] The assay used did not allow the determination of enzymic activity at these lower pH values because of the solubility of the substrate[9] Addition of rather large quantities of a crude protein preparation from the cytosol fraction did not influence enzyme activities at pH 7 2 and lower value At the optimum pH of the enzyme (7 6) and higher values, the activity decreased (*Figure 5*), which may indicate the influence of a protease inhibitor present in the cytosol[11]

The type of reaction catalysed by the protease in the of *Hevea brasiliensis* latex was investigated by using several inhibitors according to the protocol presented by Barrett[10] The enzyme was not inhibited by 3,4-dichloroiso-coumarin (3,4-DCI), which indicated that it was not a serine protease The results with inhibitors of cysteine, aspartic and metalloproteases respectively, did not assign definitely the type of reaction, but pepstatin

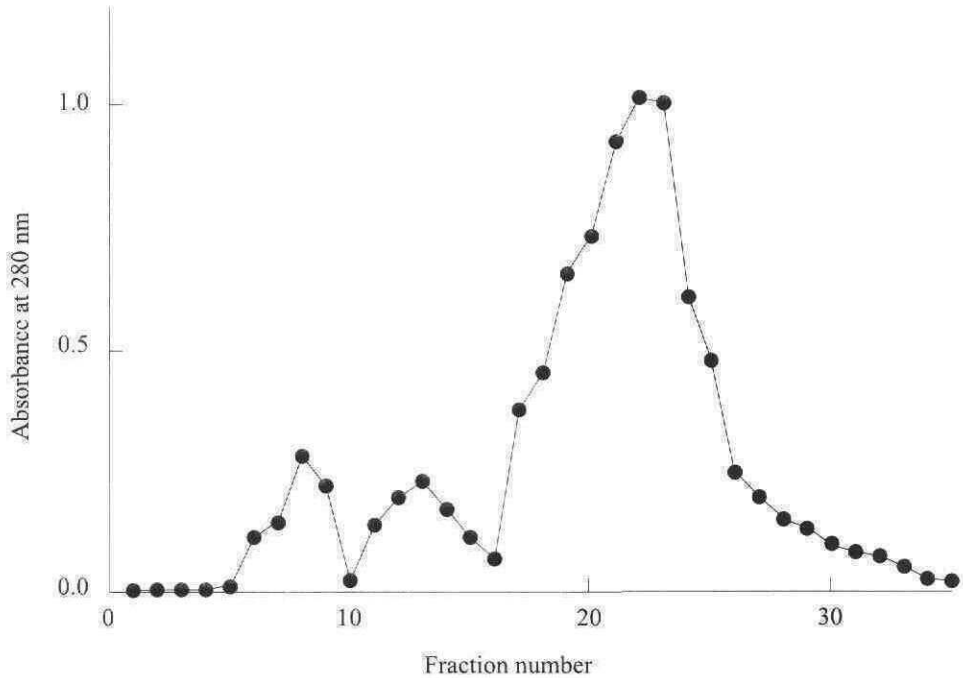


*Figure 1. Gel-filtration on a column (40 × 1.6 cm) of Ultragel AcA34 of acidic lutoid-body fraction proteins (28 Mg). Elution with 0.1 M phosphate buffer, pH 7.6. The flow rate was 100 mL.h$^{-1}$ and 2 mL fractions were collected. ( ● ): Absorbance at 280 nm ($A_{280}$). Only fractions 6-9 (first peak) exhibit protease activity.*

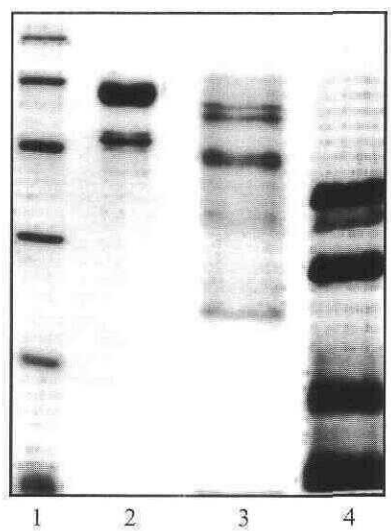


*Figure 2. SDS-PAGE on a 15 % gel of the three peaks from* Figure 1. *Lane 1: Standard proteins with $M_W$ values of 94, 67, 43, 30, 20 and 14; Lane 2: First peak; Lane 3: Second peak; Lane 4: Third peak.*

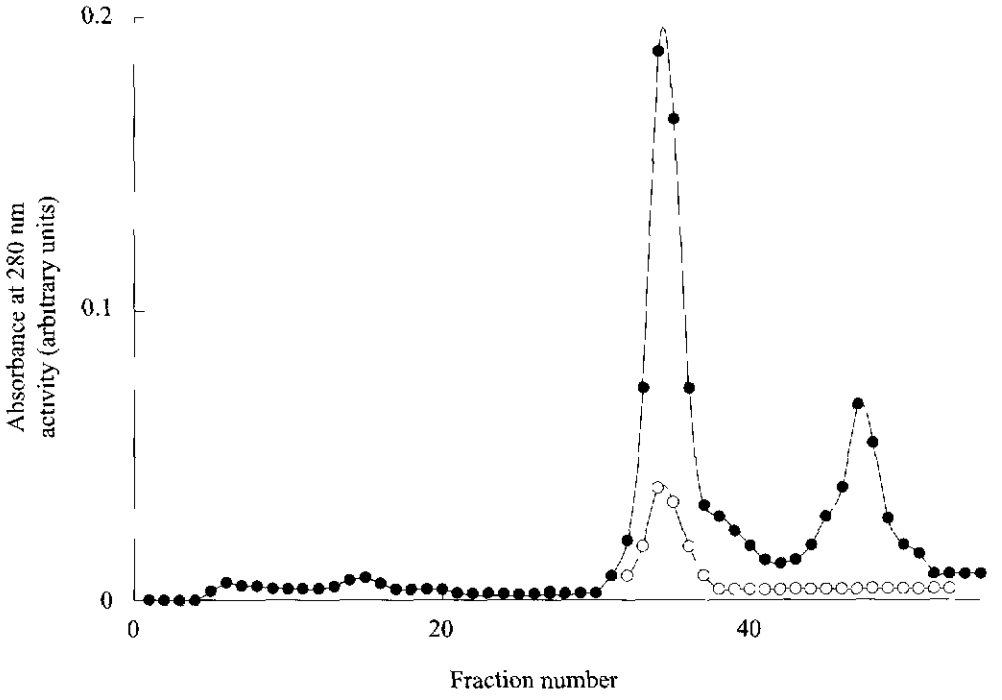


*Figure 3. Chromatography on a column (30 × 1.5 cm) of DEAE-cellulose DE52 of the first peak from* Figure 1 *Elution with 0.05 M Tris-HCl buffer, pH 8.0 with a gradient of 0-0.1 M NaCl. The flow rate was 60 mL $h^{-1}$ and 5 mL fractions were collected. (●) · Absorbance at 280 nm (A280); (○). Protease activity (arbitrary units).*

gave the strongest inhibition, suggesting that the protease was an aspartic endopeptidase (EC 3.4.23) with a relatively high pH optimum.

Earlier reports on the presence of proteases in rubber latex are those of Pujarsnicle[17], who demonstrated a hemoglobin-degrading activity in a preparation of lutoid-body proteins with maximum activity at pH 3.6 and much lower activities at higher pH values (five times lower at pH 5.5), and of Lynn and Clevette-Radford[18]. The latter authors described the isolation of a protease 'hevain' from the aquous phase of ammoniated latex, which originates from the cytosol and the lutoid-body fraction. This enzyme had a pH optimum in the range of 6.5 – 7.5 and a molecular mass of 69 kDa, but shows five protein bands ranging from 18 kDa – 60 kDa upon SDS-PAGE (which may be ascribed to degradation). In a later publication[19] these authors describe the isolation of this protease from the cytosol fraction of rubber latex, and as it is a serine protease, it is not likely that the 65 kDa protease isolated by us was identical to hevain.

The authors have not obtained evidence that the 65 kDa protease in the lutoid-body fraction was involved in the processing of the 19 kDa precursor of hevein. But there are


*Figure 4. SDS-PAGE on a 15 % gel of the purified protease (67 kDa) from the lutoid-body fractions of* Hevea brasiliensis *latex (lane 1). Lane 2, standard proteins (see legend,* Figure 2*).*

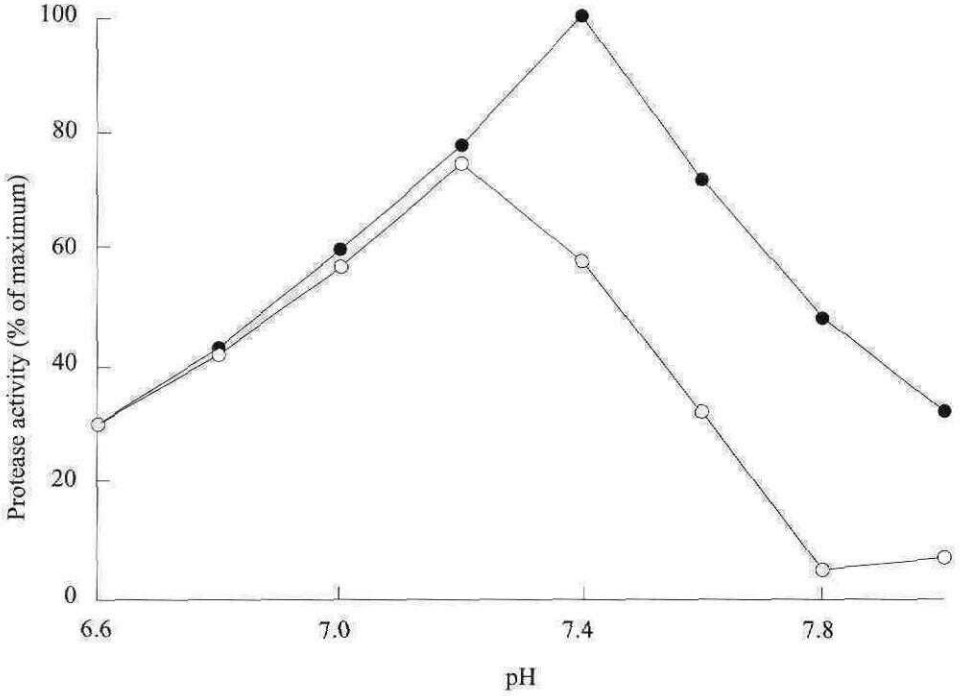


*Figure 5. Protease activity as a function of pH in 0.1 M sodium phosphate buffer of the purified protease from the lutoid-body fraction of* Hevea brasiliensis *in the absence (●) and presence (○) of a cytosolic preparation containing a protease inhibitor.*

some very preliminary indications for this. The 19 kDa band of this protein is a predominant one after SDS-PAGE of lutoid-body preparations dissolved in water[3,12] and of the first peak obtained after gel filtration of lyophilised lutoid-body fraction in 0.2 M acetic acid on Sephadex G25 at room temperature (*Figure 6A*; lane 2, arrow). When 0.2 M sodium acetate buffer, pH 6.0, was used as eluant under the same conditions, the intensity of the 19 kDa band was already lower (*Figure 6A*; lane 3; *Figure 6B*; lane 1). After dialysis and lyophilisation of a sample of the first peak of the latter chromatography, followed by incubation for one or several hours in Tris-HCl buffer, pH 7.0, the 19 kDa band had disappeared completely (*Figure 6B*; lane 2).

B-serum was produced by either repeated freeze-thawing or rigorous extraction of lutoid bodies with a homogeniser and followed by centrifugation. Both methods are used for the preparation of starting material for studies on lutoid-body proteins and there are no differences in the relative intensities of the 19 kDa hevein precursor and of the 14 kDa C-terminal fragment of the precursor after SDS-PAGE[4,5,12,13]. When low pH values were used in the initial steps for isolation of proteins, the 19 kDa band did not diminish in intensity. Evidently the 19 kDa protein was

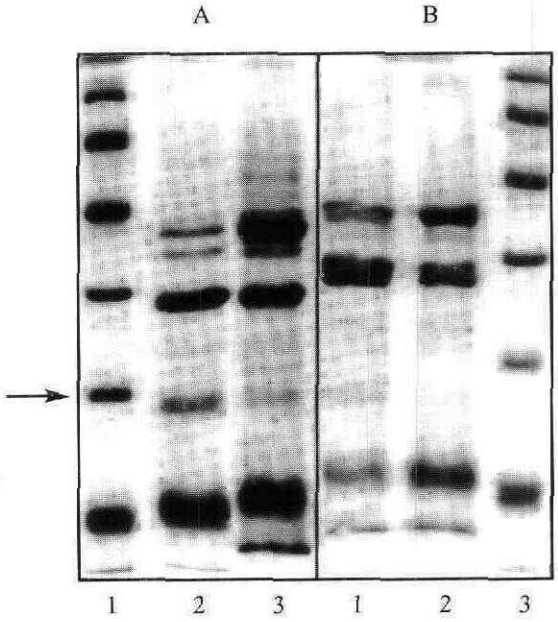


*Figure 6. SDS-PAGE on 15 % gel of mixtures of lutoid-body fraction proteins from the first peak obtained after gel filtration on Sephadex G-25. A: lane 1, standard proteins (see legend,* Figure 2*); lane 2, after gel filtration in 0.2 M acetic acid; lane 3, after gel filtration in 0.2 M sodium acetate, pH 6.0. B: lane 1, same as lane A3 (about four times smaller quantity); lane 2, as lane 1, after dialysis, lyophilisation, and incubation in 200 mM Tris-HCl buffer, pH 7.0, for 1 h; lane 3, standard proteins. Arrow, indicates the position of the 19 kDa hevein precursor.*

stable under these conditions also during the freezing/thawing procedure used to produce B-serum, because of the low pH of the lutoid-body contents However, this protein was less stable during incubations at higher pH values (*Figure 6B*)

A similar processing as that of hevein, occurs in the formation of a lectin from *Urtica dioica* (stinging nettle), which is a protein consisting of two lectin (hevein-like) domains in tandem and which is formed by proteolytic processing from a larger precursor with a family 19 chitinase domain at its C-terminus[20] The precursors of hevein and this lectin both possess a C-terminal vacuolar targeting signal, which are not present anymore in the mature proteins[5,21] Does *et al*[21] have expressed the *Urtica dioica* lectin cDNA in tobacco with and without the coding region for the C-terminal vacuolar targeting signal and observed the expected vacuolar and extracellular locations, respectively, of the expressed proteins A very interesting observation by these authors is that in both products cleavage between the lectin domains and the C-terminal residue had already occurred This means that this proteolytic digestion occurs in the cell on the way from the site of biosynthesis at the rough endoplasmic reticulum and folding of the nascent protein, before the bifurcation of transport to the vacuoles or to an extracellular destination The pH is probably still in the neutral range, allowing proteolysis In the vacuolar environment this process is prevented by lowering of the pH It will be interesting to investigate if neutral proteases with a molecular mass of about 65 kDa are also involved in removal of C-terminal targeting sequences in the generation of plant vacuolar proteins[22] Investigation of the X-ray structure of hevamine, the chitinase present in the lutoid-body fraction of rubber latex also lead to the conclusion that processing is an early step after synthesis and folding of the polypeptide chain There is a salt bridge between the N-terminus and the C-terminus of hevamine, which is important for stabilisation of its polypeptide fold [3] This salt bridge can only be formed after cleavage and removal of a C-terminal targeting sequence[24]

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REFERENCES

1 MOIR G F J (1959) Ultracentrifugation and Staining of *Hevea* Latex *Nature* **184**, 1626–1628

2 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**, 258–264

3 BROEKAERT, W J, 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 USA*, **87**, 7633–7637

4 LEE, H I, BROEKAERT W AND RAIKHEL, N V (1991) Co- and post-translational

Processing of the Hevein Pre-proprotein of Latex of the Rubber Tree (*Hevea brasiliensis*) *J Biol Chem* **266**, 15944–15948

5 SOEDJANAATNADJA, 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**, 211–213

6 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 Biophys Acta*, **1209** 144–148

7 GOMEZ, J B AND TATA, S J (1977) Further Studies on the Occurrence and Distribution of Microhelices in Clones of *Hevea J Rubber Res Inst Malaysia*, **25**, 120–126

8 LAEMMLI, U K (1970) Cleavage of Structural Proteins during the Assembly of the Head of Bacteriophage T4 *Nature* **227**, 680–685

9 REIMERDES, E H AND KLOSTERMEYER, H (1976) Determination of Proteolytic Activities on Casein Substrates (*Colowick, S P and Kaplan N O eds*), *Methods in Enzymology* (*Lorand, L ed*), Vol **45B,** pp 26–28 New York Academic Press

10 BARRETT, A J (1994) Classification of Peptidases (*Colowick S P and Kaplan, N O eds*), *Methods in Enzymology* (*Barrett, A J Ed*), vol **244** pp 1–15 New York Academic Press

11 ARCHER, B L (1983) An Alkaline Protease Inhibitor from *Hevea brasiliensis* Latex *Phytochemistry*, **22**, 633–639

12 SUBROTO T, VAN KONINGSVELD, G A, 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** 29–37

13 SUBROTO T DE VRIES H, SCHURINGA, J J, SOEDJANAATMADJA, U M S, HOFSTEENGE J, JEKEL, P A AND BEINTEMA, J J (2001) Enzymic and Structural Studies on Processed Proteins from the Vacuolar (Lutoid-body) Fraction of Latex of *Hevea brasiliensis Plant Physiol Biochem*, **39**, 1047–1055

14 SUNDERASAN, E, HAMZAH, S, HAMID, S, WARD, M A, YEANG, H Y AND CARDOSA, M J (1995) Latex B-serum β-1,3-glucanase (*Hev b* II) and a Component of the Microhelix (*Hev b* IV) are Major Latex Allergens *J Nat Rubber Res* **10(2)**, 82–99

15 BEEZHOLD, D H, SUSSMAN, G L, LISS, G M AND CHANG, N-S (1996) Latex Allergy Can Induce Clinical Reactions to Specific Foods *Clin Exp Allergy*, **26**, 416–422

16 MARIN, B, MARIN-LANZA, M AND KOMOR, E (1981) The Protonmotive Potential Difference Across the Vacuolysosomal Membrane of *Hevea brasiliensis* (Rubber tree) and its Modification by a Membrane-bound Adenosine Triphosphatase *Biochem J*, **198**, 365–372

17 PUJARSNICLE, S (1968) Caractere Lysosomal des Lutoides du Latex d'*Hevea brasiliensis* Mul-Arg *Physiol Veg*, **6**, 27–46

18 LYNN, K R AND CLEVETTE-RADFORD, N A (1984) Purification and Characterization of Hevain, a Serine Protease from *Hevea brasiliensis Phytochemistry*, **23**, 963–964

19 LYNN, K R AND CLEVETTE-RADFORD, N A (1986) Hevains Serine-centred Proteases from the Latex of *Hevea brasiliensis Phytochemistry*, **25**, 2279–2282

20 LERNER, D R AND RAIKHEL, N V (1992) The Gene for Stinging Nettle Lectin (*Urtica dioica*) Agglutinin Encodes Both a Lectin and a Chitinase *J Biol Chem* **267**, 11085–11091

21 DOFS, M P, HOUTERMAN, P M DEKKER, H L AND CORNELISSEN, B J C (1999) Processing, Targeting, and Antifungal Activity of Stinging Nettle Agglutinin in Transgenic Tobacco *Plant Physiol* **120** 421–431

22 NEUHAUS, J -M AND ROGERS, J C (1998) Sorting of Proteins to Vacuoles in Plant Cells *Plant Mol Biol*, **38**, 127–144

23 TERWISSCHA VAN SCHELTINGA A C, HENNIG, M AND DIJKSTRA, B W (1996) The 1 8 Å Resolution Structure of Hevamine, a Plant Chitinase Lysozyme and Analysis of the Conserved Sequence and Structure Motifs of Glycosyl Hydrolase Family 18 *J Mol Biol*, **262**, 243–257

24 BOKMA, E, SPIERING, M, CHOW, K S MULDER P P M F A, SUBROTO, T, AND BEINTEMA, J J (2001) Determination of cDNA and Genomic DNA Sequences of Hevamine a Chitinase from the Rubber Tree *Hevea brasiliensis Plant Physiol Biochem*, **39** 367–376