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CHITINASE AND β-1,3-GLUCANASE IN THE LUTOID-BODY FRACTION OF *HEVEA* LATEX

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Key Word Index—*Hevea brasiliensis*; Euphorbiaceae; rubber latex; chitinase; β-1,3-glucanase; latex destabilization.

Abstract—The lutoid-body (bottom) fraction of latex from the rubber tree (*Hevea brasiliensis*) contains a limited number of major proteins. These are, besides the chitin-binding protein hevein, its precursor and the C-terminal fragment of this precursor, proteins with enzymic activities: three hevamine components, which are basic, vacuolar, chitinases with lysozyme activity, and a β-1,3-glucanase. Lutoid-body fractions from three rubber-tree clones differed in their contents of these enzyme proteins. The hevamine components and glucanase were isolated and several enzymic and structural properties were investigated. These enzymes are basic proteins and cause coagulation of the negatively charged rubber particles. The coagulation occurs in a rather narrow range of ratios of added protein to rubber particles, which indicates that charge neutralization is the determining factor. Differences in coagulation of rubber particles by lutoid-body fractions from various rubber clones can be explained by their content of hevamine and glucanase. Glucanase from the lutoid-body fraction may dissolve callus tissue and this may explain the observation that rubber-tree clones with a high glucanase content in this fraction produce more latex than clones with little glucanase. Sequence studies of two CNBr peptides of the glucanase indicate that this protein is homologous with glucanases from other plants, and that a C-terminal peptide, possibly involved in vacuolar targeting, may have been cleaved off.

INTRODUCTION

Latex of the rubber tree is the cytoplasm of specialized cells known as laticifers. The major components of latex are rubber particles, cytosol and organelles called lutoids. The latter are of vacuolar origin and contain several hydrolases [1] and PR(pathogenesis-related)-like proteins [2]. Upon centrifugation, rubber latex is divided into a layer of rubber particles, the cytosol, and the lutoid-body fraction [3]. The latter fraction is composed mainly of lutoids, but may also contain other organelles, such as some mitochondria, ribosomes and rubber particles with higher density.

The first protein isolated from rubber latex was hevein, a small cystine-rich protein [4], which is formed by proteolytic processing [5, 6] of a precursor protein [7]. Two basic proteins hevamine A and B have been isolated by Archer [8] from rubber latex. Tata *et al.* [9], using a similar procedure, isolated the same proteins and demonstrated that they are bifunctional lysozymes/chitinases. The primary structures of the proteins have been determined [10]. Hevamine B differs from hevamine A in the replacement of leucine at position 270 by arginine. The proteins can be classified as basic, vacuolar family 18 [11] or Class III [2] chitinases, with no sequence homology with the family 19 [11] or Class I, II and IV [2] chitinases. The three-dimensional structure of hevamine A has been published recently [12]. However, the enzymes have not yet been well characterized further.

Lee *et al.* [5] presented a pattern of proteins separated by gel electrophoresis in the presence of sodium dodecylsulphate in which seven major bands are discernible. The major protein is hevein, which has been reported to make up the greater part of the lutoid-body fraction proteins [13]. The isolation of hevein, the processed precursor of hevein and its C-terminal fragment have been described elsewhere [6, 14]. Here we describe the isolation and further characterization of the two major enzymic activities in the lutoid-body fraction, viz. the already discussed lysozyme/chitinases and a β-1,3-glucanase, several of their enzymic properties,

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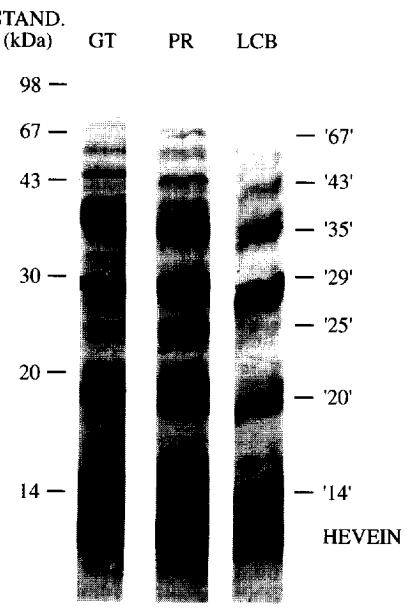

Fig. 1. SDS-PAGE on a 15% gel of *ca* 50 μg quantities of lutoid-body fractions of clones GT.1, PR 261 and LCB 1320. The left lane indicates standard proteins with known M_rs in kDa. To the right, the positions of hevein and several other lutoid-body proteins are indicated with their M_rs in kDa.

and the destabilizing effect these basic proteins have on a suspension of rubber particles.

RESULTS AND DISCUSSION

From 1 l of rubber latex, about 150 ml of lutoid-body fraction were obtained, yielding 5–10 g lyophilized material. The protein patterns of preparations from the three investigated rubber clones after SDS-PAGE are presented in Fig. 1. Against a background of minor proteins, five major bands are visible. These are hevein (M_r 5000), the C-terminal fragment of prohevein (M_r 14 000) prohevein itself (M_r about 20 000) [5], and two bands with M_rs 29 000 and 35 000, respectively. Less abundant bands were present in varying amounts. There were differences between the clones: the M_r 35 000 band was most dense in clone GT.1, and very weak in clone LCB 1320. The M_r 35 000 band has a slightly lower mobility in clone GT. 1 than in clone PR 261. Generally, the intensity of this band was greater in preparations from lyophilized lutoid-body fractions, than in those obtained by repeated freezing and thawing of this fraction [15]. Lee *et al.* [5] found protein bands with similar molecular masses in clone RRIM 600 and, in addition, major bands of a M_r 25 000 and a M_r 67 000 protein. In the three clones shown in Fig. 1, only in clone PR 261 may the M_r 25 000 protein-band be considered a major one. We also found a predominant M_r 43 000 band in all three clones. This band is more easily seen after silver staining, than after staining with Coomassie Brilliant Blue.

Chitinase

After ammonium sulphate precipitation and gel filtration to separate hevein from the other proteins, the latter were subjected to column chromatography on carboxymethylcellulose CM32. Before starting the chromatography, the protein solution was dialysed against the buffer with which the column was equilibrated. This treatment resulted in a rather large precipitate, in which the M_r 35 000 protein was the major component. The other major proteins remained soluble, and were separated by column chromatography on carboxymethylcellulose (Fig. 2). Prohevein and its C-terminal fragment were the major components of peaks

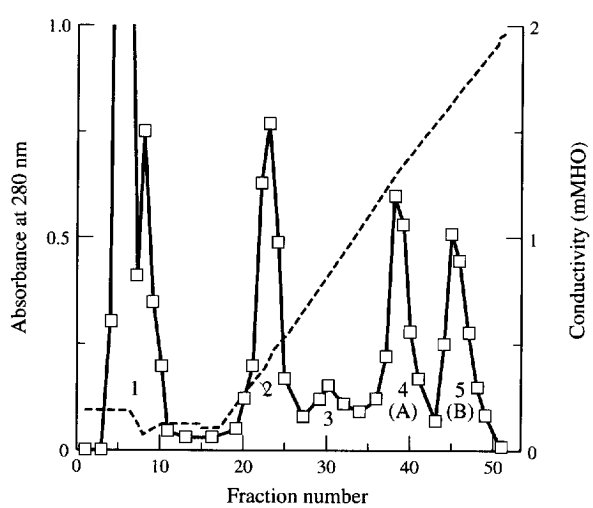

Fig. 2. Chromatography of 1 g of lyophilized B serum of clone GT.1, after removal of hevein by gel filtration, on a carboxymethylcellulose CM32 column (1.5 × 20 cm). Elution with a linear gradient of 0.04–0.4 M borate buffer, pH 8.9. Fractions of 2 ml were collected. (A) and (B) indicate the peaks of hevamine A and B, respectively. —□—, absorbance at 280 nm; ----, conductivity.

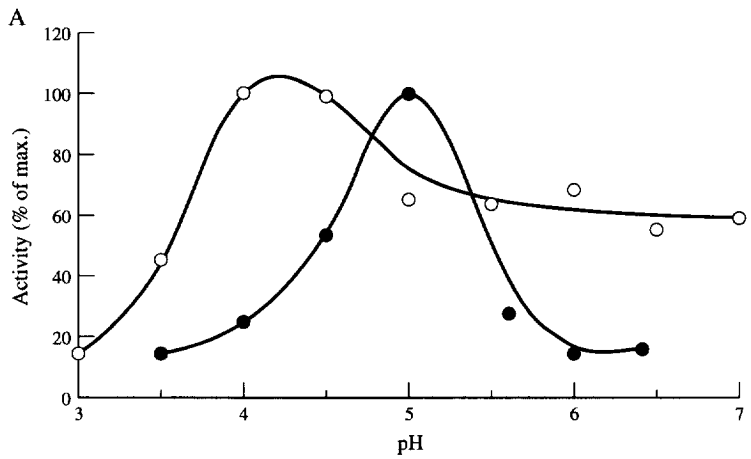

Fig. 3A. Lysozyme (●) and chitinase (○) activities of hevamine A as functions of pH. Activities are indicated as % of the maximum. 0.005 M (lysozyme) or 0.1 M (chitinase) sodium acetate (pH 3.5–5.5) and Tris-acetate (pH 5.5–6.5) buffers were used. Lysozyme activities were determined as described in ref. [9], and chitinase activities as described in ref. [19]. If the chitinase activity is plotted as a function of hevamine concentration, a hyperbolic curve is obtained. Activities were determined from this curve.

2 and 1, respectively [6]. Proteins with a M_r of 29 000 eluted in peaks 3, 4 and 5, and had chitinase and lysozyme activity, with similar specific activities (data not shown). Peaks 4 and 5 contained pure hevamine A and B [8], respectively. Quantities of 2–3 mg of each were obtained per g of lyophilized lutoid-body fraction. *N*-terminal sequence analysis of the minor peak 3 showed that it contained a third, less basic, hevamine component. Recently, Punya *et al.* [16] also reported the presence of three chitinase components in the lutoid-body fraction of rubber latex with pI values of 9.3, 9.7, and 9.8, respectively. Martin [17] described the separation of seven chitinases from the lutoid-body fraction of rubber latex. It is not possible to relate these results with those described here and in [8, 9 and 16].

Tata *et al.* [9] described the lysozyme activity of hevamine A and B. Later, Bernasconi *et al.* [18] presented the *N*-terminal sequence of a chitinase/lysozyme from *Parthenocissus quinquifolia* and several properties of this enzyme. As its *N*-terminal sequence differs at only 3 out of 47 positions from that of hevamine [10], we expected that the enzymic properties of both enzymes would also be very similar. Fig. 3A presents the pH optima of the lysozyme and chitinase activities of hexamine, and Fig. 3B the dependence of these activities on salt concentration. We found that hevamine has a specific lysozyme activity similar to that of hen-egg-white lysozyme, although Tata *et al.* [9] found a value five times lower. The lysozyme activity of hevamine has a sharp optimum at pH 4.9, while that of the chitinase activity extends over a much broader range and extends from pH 4 to higher than pH 6. Lysozyme activity can be observed only at low salt concentrations, while the chitinase activity is much less

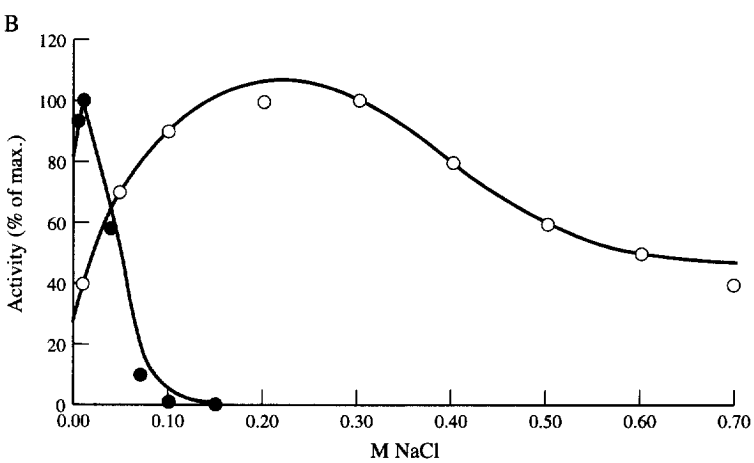

Fig. 3B. Lysozyme (●) and chitinase (○) activities of hevamine A as functions of the salt concentration. Activities are indicated as % of the maximum. The buffer used was 0.01 M sodium acetate, pH 5.2 (chitinase) or 0.005 M sodium acetate, pH 4.9 (lysozyme), with addition of increasing amounts of NaCl.

dependent on salt concentration. These features are not only very similar to those observed for the *Parthenocissus* enzyme, but also to those described for the structurally unrelated basic chitinase/lysozyme from bean [20] and the basic lysozyme from papaya latex [21]. This suggests that the negative charges in lysozyme substrates are essential for interaction with basic plant lysozyme/chitinases and that these interactions occur only at low pH values and salt concentrations.

Archer [8] found similar quantities of hevamine A and B, but Tata *et al.* [9] reported a higher relative recovery of hevamine A. We found that the two variants occur in about equal quantities in clone GT.1 (Fig. 2), but that there may be relatively less hevamine B in clone PR 261. A surprising result was that clone LCB contained no hevamine B. Only the peak of hevamine A and the small peak in front of it were observed (data not shown).

Hevamine belongs to the family 18 chitinases, which have no sequence or other structural similarity to the family 19 chitinases [11]. Martin [17] reported that *Hevea* chitinases cross-react with an antibody against a class 19 chitinase and Gidrol *et al.* [22] found the expression of such a chitinase by Northern-blot analysis. However, so far we have found only family 18 chitinases in the lutoid-body fraction of rubber latex.

B-1,3-*Glucanase*

The precipitate obtained from an extract of lyophilized lutoid-body proteins from clone RRIM 600 at 65% saturation with ammonium sulphate, was dialysed and submitted to ion-exchange chromatography on a column of carboxymethylcellulose in 0.1 M Tris buffer, pH 8.0., with a gradient of 0–0.5 M NaCl (data not shown). Only one major protein was eluted after application of the salt gradient. A crystalline precipitate had formed in several tubes after standing in the cold room for one to two weeks. After desalting on Sephadex G-25 and lyophilization, a protein with a M_r of 35 000 was obtained in a recovery of about 4.5 mg per g lyophilized lutoid-body fraction.

Sequence analysis indicated a blocked *N*-terminus. After cleavage with CNBr and reversed-phase HPLC of the soluble part of the digest, a peptide mixture consisting of a major and a minor component was obtained. The *N*-terminal amino acid sequences of these peptides are present in Fig. 4. Comparison with amino acid sequences in a protein sequence database (Eurosequence BV, Groningen) revealed more than 50% sequence identity of both peptides with members of the family of β-1,3-glucanases (PR-2 proteins). The major peptide is homologous with the *C*-terminal part of these proteins and probably has a *C*-terminal Phe-Gly sequence. The minor peptide is homologous with a more *N*-terminally located sequence. Fig. 4 shows the homology with sequences of a basic tobacco enzyme. The amino acid composition of the M_r 35 000 protein is also similar to those of other plant glucanases, including a low cysteine content. These findings indicate that the M_r 35 000 protein may represent a mature basic, vacuolar β-1,3-glucanase with a cyclized *N*-terminal Gln and a cleaved-off *C*-terminal sequence [24]. However, as the final step of the isolation procedure caused denaturation of the protein, we have not been able to confirm our assignment by demonstration of enzymic activity with this preparation.

Later efforts to repeat the chromatography on carboxymethylcellulose CM32 in 0.1 M Tris buffer, pH 8.0, to isolate the M_r 35 000 protein were unsuccessful, because most of it precipitated during the dialysis step, and only a small amount was obtained from the carboxymethylcellulose column. Therefore, a lower pH value was chosen for the isolation of this protein on carboxymethylcellulose CM32. Fig. 5 presents the elution pattern of lyophilized lutoid-body fraction proteins from clone PR 261 after extraction, 65% saturation with ammonium sulphate, dialysis against 40 mM sodium acetate pH 5.2 (with formation of very little precipitate), and chromatography in the same acetate buffer with a gradient of 0–0.4 M NaCl. Pure M_r 35 000 protein was obtained in a high recovery. We found that only the M_r 35 000 protein peak had glucanase activity, with an activity of about 150 nkat mg^{-1} protein [25], while peaks with chitinase activity eluted earlier. About 2 mg β-1,3-glucanase were obtained per g lyophilized lutoid-body fraction from clone PR-261. The recovery from clone GT.1 was about three times higher, while that from clone LCB 1320 was negligible. These recoveries are in agreement with the gel electrophoresis patterns of the three clones presented in Fig. 1. The purified glucanase from clone PR 261 had again a slightly lower apparent M_r than that from clone GT.1.

Meanwhile, Churngchow *et al.* [26] and Breton *et al.* [27] have also described the presence of basic β-1,3-glucanases in the lutoid-body fraction of rubber latex. They also found two isozymes with similar M_r (32 000 and 35 000, respectively, according to [26]) in clones RRIM 600 [26] and PB 86 and PB 235 [27], and also relatively high β-1,3-glucanase contents of the M_r 35 000 isoenzyme in clone GT.1. However, Breton *et al.* [27] also describe the presence of acidic β-1,3-glucanases, which could not be demonstrated in our work (Fig. 5). Churngchow *et al.* [26] have demonstrated that the M_r 35 000 isoenzyme is a glycoprotein, which may explain its higher apparent M_r on gel electrophoresis.

We have performed some preliminary sequence studies on CNBr digests of the β-1,3-glucanases from clones GT.1 and PR 261. The SDS-PAGE patterns of the two digests were different (Fig. 6). While the digests of glucanases from clones RRIM 600 and PR 261 were quite insoluble, that from clone GT.1 dissolved easily. These results indicated that the enzyme from the latter clone may have a deviating sequence. Peptides starting at the homologous position 309 of tobacco glucanase (see Fig. 4) were isolated and sequenced. The peptide from clone PR 261 was identical to that from clone RRIM 600. Its M_r found by

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                31              35                  40
TOBACCO        -Met-Leu-Gly-Asn-Asn-Leu-Pro-Asn-His-Trp-Glu-Val-Ile-Gln-
RUBBER TREE     ___-Gln-Gly-Asn-Xxx-Leu-Pro-Pro-Val-Ser-Glu-Val-Ile-Ala-
pmol               227 193 200     225 180 156 128  66  +   +  102  77

                45                  50
TOBACCO        -Leu-Tyr-Lys-Ser-Arg-Asn-Ile-
RUBBER TREE    -Leu-Tyr-Lys-Lys-Ser-Xxx-Ile-
pmol            96  61  38  57  35      58

                308     310                 315                 320
TOBACCO        -Met-Phe-Asp-Glu-Asn-Asn-Lys-Asn-Pro-Glu-Leu-Glu-Lys-His-
RUBBER TREE     ___-Phe-Asp-Glu-Asn-Lys-Lys-Gln-Pro-Glu-Val-Glu-Lys-His-
pmol              1434 407 500 857 683 663 512 462 250 416 204 300  47

                            325                 330                 335
TOBACCO        -Phe-Gly-Leu-Phe-Ser-Pro-Asn-Lys-Gln-Pro-Lys-Tyr-Asn-Leu-
RUBBER TREE    -Phe-Gly-Leu-Phe-Phe-Pro-Asp-Lys-Xxx-Gln-Lys-Tyr-Asn-Leu-
pmol            305 189 316 287 348 174 104  62      98  40  61  83  80

                        338
TOBACCO        -Asn-Phe-Gly
RUBBER TREE    -Asn-Phe-Gly___
pmol            97  50  19
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Fig. 4. Amino acid sequences of two CNBr peptides of the 35 kDa protein of clone RRIM 600, aligned with homologous sequences of the mature form of a β-1,3-glucanase from tobacco [23,24]. Identities in the tobacco and *Hevea* enzymes are underlined. Recoveries of amino acid derivatives (in pmol) are presented below the sequences. Xxx, unidentified residues.

ion-spray mass spectrometry was 3747, but assignment of the unknown residue at position 330 is not yet possible. The peptide from clone GT.1 differed from those of the two other clones at positions 328 (Asn), 336 (Leu), and 338 (Ser).

Other lutoid-body fraction proteins

The unretarded peak 1 from the CM-cellulose chromatography (Fig. 2) was used to isolate acidic proteins from the lutoid-body fraction of rubber latex by chro-

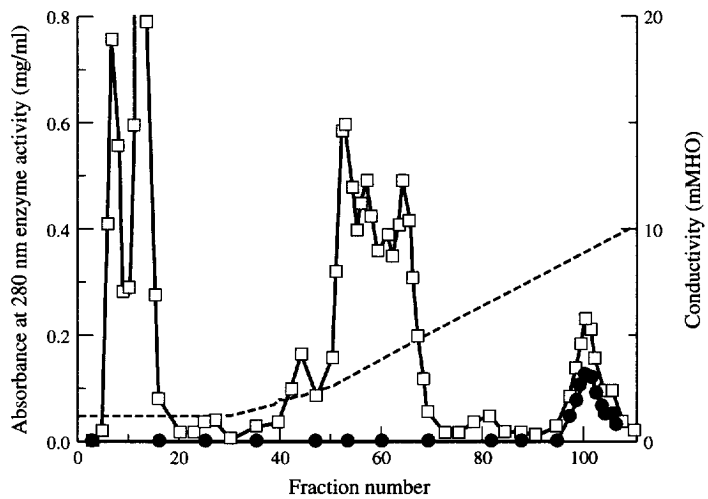

Fig. 5. Chromatography of the 65% saturated ammonium sulphate of an extract of 500 mg lyophilized lutoid-body fraction of clone PR 261 on a carboxymethylcellulose CM32 column (1.5 × 20 cm). Elution with a linear gradient of 0.0–0.4 M NaCl gradient in 40 mM sodium acetate buffer, pH 5.2. Fractions of 2 ml were collected. —□—, absorbance at 280 nm; —●—, glucanase activity (mg ml^{-1}); ----, conductivity.

matography on DEAE-cellulose. A pure protein with a M_r of 43 000 was obtained. Preliminary sequence studies were performed to characterize this protein. Beezhold *et al.* [28] have performed similar studies, and found that the *N*-terminal amino acid sequence and that of an internal CNBr peptide (total 29 residues) are about 60% identical with sequences of patatin. This is the major storage protein from potato tubers, and is a glycoprotein of 363 amino acid residues, with an apparent M_r of 40 000–43 000 [29]. Contrary to Beezhold *et al.* [28], we found a blocked *N*-terminal sequence in the M_r 43 000 protein isolated by us. However, a short internal CNBr peptide has also about 60% sequence identity with a patatin sequence, as presented below (underlined are identical residues):

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PATATIN:       Met-Ile-Thr-Thr-Pro-Asn-Glu-
RUBBER TREE:   -Leu-Thr-Ala-Pro-Asn-Glu-
PATATIN:       Thr-Asn-Arg-Pro-Phe-
RUBBER TREE:   -Asp-Lys-Lys-Pro-Met
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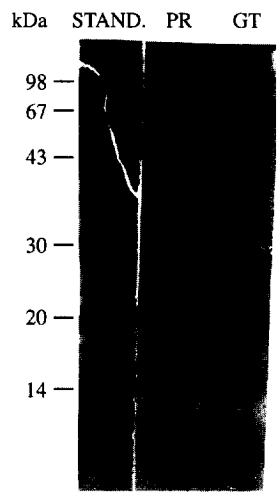

Fig. 6. SPS-PAGE on a 15% gel of CNBr digests of β-1,3-glucanase from clones PR 261 and GT.1. The left lane indicates standard proteins with known M_r in kDa.

Coagulation of rubber particles

Upon wounding, lutoids in rubber latex burst and cause coagulation of the latex by an interaction between the negatively charged rubber particles and cationic proteins from the lutoids [30]. Recently, Gidrol *et al.* [22] showed that hevein, which is an acidic chitin-binding protein, is also involved in the coagulation of rubber particles. We performed our experiments with freshly tapped rubber latex (clone GT.1) which was diluted 1:200 with the same buffer solution as described by Gidrol *et al.* [22], but with 0.5 mg ml^{-1} sodium dithionite replacing 0.1 mM dithiothreitol as a reducing agent. The suspension was stable for several days.

The observed extent of coagulation in the cuvettes was similar to that presented by Gidrol *et al.* [22] in figure 1B of their paper. However, our results differed in two respects. First, we observed coagulation of rubber particles only as a result of incubation with basic proteins, and not with acidic ones like bovine serum albumin or HPLC-purified [14] samples of the chitin-binding protein hevein, in similar, or much higher quantities than used by Gidrol *et al.* [22]. Second, we found that the coagulation rate as measured by the change in absorbance at 600 nm, was much more dependent on the dilution of the rubber particle suspension than on the quantity of added protein. Small quantities of added protein did not coagulate the

particles. On increasing the protein quantity, coagulation occurred, but a saturation value above which larger quantities had no additional effect was rapidly reached. Fig. 7 shows the effect of the addition of increasing quantities of β-1,3-glucanase to the diluted suspension. Quantities up to 15 μg had no effect, while the maximum effect already occurred with 25 μg. The explanation must be that the negative charges on the rubber particles are titrated by increasing amounts of a basic protein, and that at neutralization, the maximum extent of coagulation is reached. Neutralization of the rubber particles was reached by the addition of about 20 μg glucanase.

Other basic proteins like hevamine A and B, and bovine pancreatic ribonuclease, also coagulated the dilute suspension of rubber particles. However, larger quantities were needed (120–170 μg protein). Amounts of lutoid-body fractions of the three rubber clones required for coagulation of rubber particles were in agreement with their glucanase and chitinase contents. However, in the field, rubber latex from clone LCB 1320 coagulates more rapidly during tapping than latex from clones GT.1 and PR 261 (and produces a higher quality rubber; A. Darussamin, personal communication). An explanation for this may be that glucanases not only dissolve fungal glycans, but also callose in callus tissue (F. Meins, Jr., personal communication). This may lead to a longer latex flow from rubber trees with high lutoid-body glucanase contents, such as clones GT.1 and PR 261.

EXPERIMENTAL

Isolation of proteins. Rubber latex of the clones GT.1, PR 261 and LCB 1320 of *Hevea brasiliensis* was collected at several plantations in the area of Subang (Western Java, Indonesia), cooled in ice and centrifuged at high speed to collect the lutoid-body fraction [3]. This fraction was directly lyophilized or submitted to repeated freezing and thawing, followed by lyophilization [15].

Lyophilized lutoid-body fraction was homogenized (Ultra Turrax, Janke & Kunkel, Staufen, F.R.G.) at 4° in H_2O (4 mg ml^{-1}) to which 0.5 g l^{-1} Na dithionite was added to inhibit polyphenol oxidases. After centrifugation, the soln was 100% satd with $(NH_4)_2SO_4$ and centrifuged again. The ppt was dissolved in a small vol. of 0.2 M HOAc and submitted to gel filtration on Sephadex G-25. The second peak contained hevein [14]. The first peak was suspended in a small vol. of 0.04 M Na borate buffer, pH 8.9, dialysed overnight against the same buffer, and centrifuged. The supernatant was submitted to cation-exchange chromatography on a carboxymethyl-cellulose column CM32 (1.5 × 20 cm), with a gradient of 0.04–0.4 M borate buffer, pH 8.9, as described in ref. [8]. Frs containing protein were pooled, dialysed against H_2O, and lyophilized.

The unretarded material from the CM-cellulose column was dialysed against 0.1 M Tris buffer, pH 8.0, and submitted to anion-exchange chromatography on a DEAE-cellulose column (1.5 × 10 cm), with a gradient of 0–0.5 M NaCl in the same buffer.

Lyophilized lutoid-body fraction of rubber latex of clone RRIM 600, kindly supplied by Dr. S. Tata, Rubber Research Institute of Malaysia, Kuala Lumpur, Malaysia, was homogenized as described above. A ppt from the extract, obtained with $(NH_4)_2SO_4$ at 65% satn, was dissolved in 0.1 M Tris buffer, pH 8.0, containing 0.5 g l^{-1} Na dithionite. After dialysis, the soln was submitted to cation exchange chromatography on a carboxymethylcellulose CM32 column (1 × 30 cm), with a gradient of 0–0.5 M NaCl in the same buffer. The peak frs containing a M_r 35 000 protein were desalted by chromatography on a Sephadex G-25 column in 0.2 M HOAc, and lyophilized.

Ppts. obtained from extracts of homogenates of lyophilized lutoid-body fractions of clones GT.1, PR 261 and LCB 1320 with $(NH_4)_2SO_4$ 65% satn, were

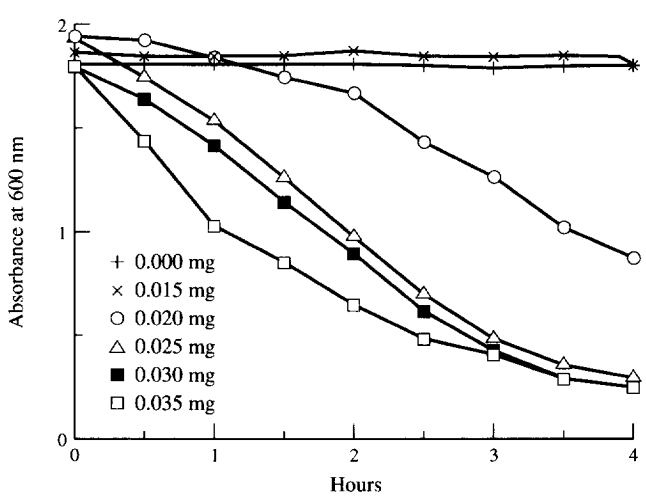

Fig. 7. Coagulation of a rubber particle suspension by increasing amounts of β-1,3-glucanase. The change in A at 600 nm at different time intervals was measured.

dissolved in 40 mM NaOAc buffer, pH 5.2, containing 0.5 g l^{-1} dithionite. After dialysis, the soln was submitted to cation exchange chromatography on a carboxymethylcellulose CM32 column (1.5 × 20 cm), with a gradient of 0–0.4 M NaCl in the same buffer.

Methods of protein and enzyme analysis. SDS-PAGE was performed on 15% gels using the discontinuous buffer system of ref. [31]. Lysozyme activities were determined as described in ref. [9], chitinase activities as described in ref. [19], and β-1,3-glucanase activities as described in ref. [25], with determination of the produced glucose according to ref. [32].

Coagulation studies on rubber latex were performed as described in ref. [22]. The rubber layer from freshly-tapped latex from clone GT.1 (collected in a rubber plantation in Cikalong Wetan, Western Java, Indonesia) was obtained by centrifugation [3]. It was diluted 1:200 in 20 mM Tris-HCl buffer, pH 6.8, 0.2 M mannitol, 0.05 mM $MgCl_2$, 0.1 mM $CaCl_2$, to which 0.5 g l^{-1} Na dithionite was added. Only the reducing agent and its concn, differed from the 0.1 mM dithiothreitol used before [22]. Small vols of protein solns were added to 1 ml quantities of the dilute rubber suspension (2–4 mg ml^{-1}), and the change of A at 600 nm monitored as a function of time. Using diluted rubber layer or diluted whole latex made not much difference to the results.

The amino acid composition of the M_r 35 000 protein was determined after acid hydrolysis in 6 M HCl at 110° for 24 hr by analysis with a Kontron Liquimat III amino acid analyser. Several protein samples were cleaved with CNBr (50 mg mg protein^{-1}) in 70% formic acid (0.1 ml mg protein^{-1}) at room temp. for 24 hr. After digestion, the cleavage mixtures were lyophilized several times from H_2O, and suspended in 0.2 M NH_4HCO_3. Peptides were isolated from soluble parts of the digests by reversed-phase HPLC on a nucleosil 10 C18 column (30 × 0.45 cm), with a gradient of 0–70% acetonitrile in 0.1% CF_3COOH for 60 min, at a flow rate of 1 ml min^{-1}. The effluents were monitored at 214 nm, and the peaks collected manually. *N*-terminal amino acid sequences of the M_r 35 000 protein and CNBr-peptides, were determined with an Applied Biosystems Model 477A Protein Sequencer with an on-line Model 120A PTH-Analyser (Eurosequence BV, Groningen). M_rs of peptides were determined as described earlier [14].

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