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Demonstration of β-*1,3-glucanase Activities in Lutoids of* Hevea brasiliensis *Latex*

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β-1,3-glucanase activity, generally known to be involved in plant defence reactions against pathogens, was observed in extracts of lutoids from Hevea *latex The β-1,3-glucanase activities detected after native isoelectrofocusing (IEF) led to the visualisation of five bands consisting of three basic isozymes (pI 10 00 ± 0 02, 9 32 ± 0.24 and 9 0 ± 0 22), and two acid isozymes (pI 5 49 ± 0 02 and 4 88 ± 0 03) Immunodetection with tobacco polyclonal antibodies against acid and basic β-1,3-glucanases, revealed the same protein profile, characterised by three major bands with molecular weights of 38 9 ± 1 2, 36.4 ± 0 89 and 30 1 ± 1 kD, respectively The presence of apparently constitutive chitinases and β-1, 3 glucanases in lutoids is discussed*

Latex is the cytoplasm of laticiferous cells in *Hevea brasiliensis* It contains 10% to 20% by volume polydispersed lysosomal microvacuoles called lutoids These single-membrane organelles named by Ruinen[1] contain many proteins some of which are deposited in helice form, especially in young tissue[2,3] (for recent review about lutoids, see *reference 4*) The lysosomal character of lutoids was discovered by Pujarniscle[5], who showed the presence of a large number of the acid hydrolases characteristic of animal cell lyso- somes (acid phosphatases, nucleases, proteases, mannosidases, *etc*) Acid and basic peroxidases have also been found in lutoids[6] The first electrophoretic analysis of intralutoid serum (B-serum) revealed the presence of acidic and basic proteins, the most abundant of these are hevein and hevamine[7] Hevein, an acidic protein appears to be a lectin, it can fix chitin and has antifungal properties[8,9] It has recently been shown that hevein is probably involved in latex coagulation, where it bridges rubber particles *via* a surface receptor protein which possesses N-acetylglucosamine groups[9] Hevamine is a basic protein characterised by two enzymatic activities, chitinase (EC 3 2 1 36) and lysozyme (EC 3 2 1 17)[10] This enzyme has been characterised biochemically and sequenced It displays strong homology with tobacco chitinases belonging to group-3 pathogenesis-related proteins (PRs)[11-13] Seven other chitinases have recently been identified in lutoids by two-dimensional electrophoresis They comprise six basic chitinases/lysozymes, and one acid chitinase with no lysozyme activity[14] These enzymes are capable of hydrolysing on the one hand, the chitin in fungal walls, insect exoskeletons and nematode cuticles, and on the other, the peptidoglucan in bacterial walls through their lysozyme activity Chitinases are classically elicited during host-parasite interactions Elicitation is frequently associated with that of β-1,3-glucanases (EC 3 2 1 39), which belongs to PR group – 2 β-1,3-glucanases can hydro-

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lyse polymers of the β-1,3-glucans, essential parietal components of most fungi. The research reported here was therefore aimed at seeking β-1,3-glucanase activity in the lutoids.

MATERIALS AND METHODS

Plant Material

The lutoids were obtained at the IDEFOR-DPL station at Bimbresso (Côte d'Ivoire) by ultracentrifugation of fresh latex using the method of Ribaillier *et al*[15]. They were freeze-dried and stored at –30°C.

Extraction and Appraisal of Lutoid Proteins

The extracts were prepared from lutoids from the three rubber-tree clones PB 86, PB 235 and GT 1. The freeze-dried material was ground under liquid nitrogen and then suspended (1:10 m/v) for 15 min at 4°C in 0.5 *M* Na-acetate buffer, pH 5.2, containing 15 m*M* β-mercaptoethanol[16], with agitation. After two successive centrifugations (40 000 g × 20 min), the soluble phase was recovered using a syringe and filtered through a 0.45 µm *Durapore* membrane. The quantity of protein extracted was estimated using the method of Bradford[17], with bovine albumin serum as the standard.

Assay of β-1,3-glucanase

β-1,3-glucanase activity was determined by colourimetry in 50 m*M* Na-acetate buffer, pH 5.2, using laminarin at 0.5 mg/mL (Sigma) as substrate. The released reducing sugars were assayed by the method of Dygert *et al*[18].

Isoelectrifocusing (IEF) under Native Conditions

IEF was performed using the method of Robertson *et al.*[19] on vertical 5% acrylamide minigels containing 2% ampholines. Acid and basic isozymes were separated by reverse migration on a pH 2-11 gradient of ampholines (SERVA). A mixture of pI markers ranging from 3.5 to 9.3 (Sigma), was used to determine the pI of the various isozymes according to their electrophoretic mobilities.

Detection of β-1,3-glucanase Isozymes on IEF Gels

The gel was incubated for 2 to 3 h at 37°C in 50 m*M* Na-acetate buffer, pH 5.2, to which was added 1.5 mg/mL of laminarin. It was then placed in 150 m*M* K_2HPO_4, pH 8.6, containing 0.05% aniline blue, for at least 30 min[20]. The lysis zones, visualised using a transilluminator at 366 nm, appeared as dark bands on the fluorescent background.

SDS-PAGE

Proteins were separated by electrophoresis under denaturing conditions (SDS-PAGE) on discontinuous polyacrylamide minigels using the method described by Laemmli[21]. The minigels consisted of a concentration gel with 5% acrylamide and a resolution gel with 12% acrylamide (6.5 cm × 10 cm). A mixture of protein markers (SDS-7 Sigma) was used to estimate the molecular mass of the proteins.

Immunodetection

After SDS-PAGE, the proteins were transferred to an immobilon P membrane (Millipore), using the semi-dry Milliblot TM-SDE transfer system (Millipore), with a current of 2 mA cm^{-2} for 30 min at ambient temperature with a discontinuous system of buffers of low ionic strength as recommended by the manufacturer:

- Anode buffer 1:0.3 *M* Tris, 10% methanol, pH 10.4

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- Anode buffer 2:25 m*M* Tris, 10% methanol, pH 10.4
- Cathode buffer: 25 m*M* Tris, 40 m*M* 6-aminohexanoic acid, 20% methanol, pH 9.4.

The immunoblot was revealed according to the procedure of Harlow and Lane[22]. Two polyclonal sera, anti-acid β-1,3-glucanase and anti-basic β-1,3-glucanase of tobacco, were diluted 1/2000 with 10 m*M* phosphate-buffered saline, pH 7.4 (137 m*M* NaCl, 2.7 m*M* KCl). The second antibody was an anti-rabbit immunoglobulin combined with alkaline phosphatase. Detection was with bromochloroindole phosphate and nitro-blue tetrazolium; the mauve products of the reaction were precipitated on the membrane at the site of the second antibody.

Assessment of Variability

Data represented are means obtained with seven repetitions of the extraction procedure.

RESULTS

The quantity of soluble proteins extracted was similar in the three clones *(Figure 1)*. In addition, there was no significant difference in β-1,3-glucanase activities in clones PB 86 and PB 235. The activity was slightly greater in clone GT 1 (*Figures 2a* and *2b*).

β-1,3-glucanase activity is attributable to at least five major proteins that can be differentiated according to their isoelectric point (pI) into five distinct bands of activities *(Figure 3)*. Two acid isozymes, with a pI of 5.49 ± 0.02 and 4.88 ± 0.03, respectively and three basic isozymes with pI of approximately 10.0 ± 0.2, 9.32 ± 0.24 and 9.0 ± 0.22 were found.

The immunoblot showed that both the polyclonal antibodies of tobacco display a serological cross-reaction with the lutoid β-1,3-glucanases. This serological relationship seems to be independent of the acid or basic nature of the isozymes. Indeed, the acid and basic β-1,3-glucanases appear to display common antigenic determinants recognised by the two types of antibody used separately. Immunodetection thus revealed three major protein bands with respective molecular masses of 38.9 ± 1.2 kD, 36.4 ± 0.89 kD and 30.1 ± 1 kD *(Figure 4)*.

DISCUSSION

The work shows that the lutoids of *Hevea* latex contain two acid β-1,3-glucanases characterised respectively by a pI of 5.49 ± 0.02 and 4.88 ± 0.03, and three basic isoenzymes with a pI in the neighbourhood of 10.0 ± 0.2, 9.32 ± 0.24 and 9.0 ± 0.22. The acid and basic β-1,3-glucanases of tobacco are found in different cell compartments. The acid isozymes are in the apoplast, whereas their basic counterparts are vacuolar[23,24]. However, this arrangement does not occur in all cases, showing that a low pI is not the only factor that determines protein stability under apoplastic conditions[25,26]. The presence of acid and basic isoenzymes in the same cell compartment would nevertheless seem surprising, if peroxidases and chitinases, acidic and basic, had not already been found in lutoids[6,14]. The acid and basic β-1,3-glucanases may differ in biochemical characteristics that control their enzymatic efficiency, as has been demonstrated for β-1,3-glucanases in tobacco[16,23].

The five isozymes display a serological cross-reaction with both the anti-β-1,3-glucanase antibodies of tobacco. It seems that the anti-acid β-1,3-glucanase and anti-basic β-1,3-glucanase antibodies are not specific to the acidic and basic nature of the different lutoid isozymes, when tested separately. This serological relationship has also been observed in

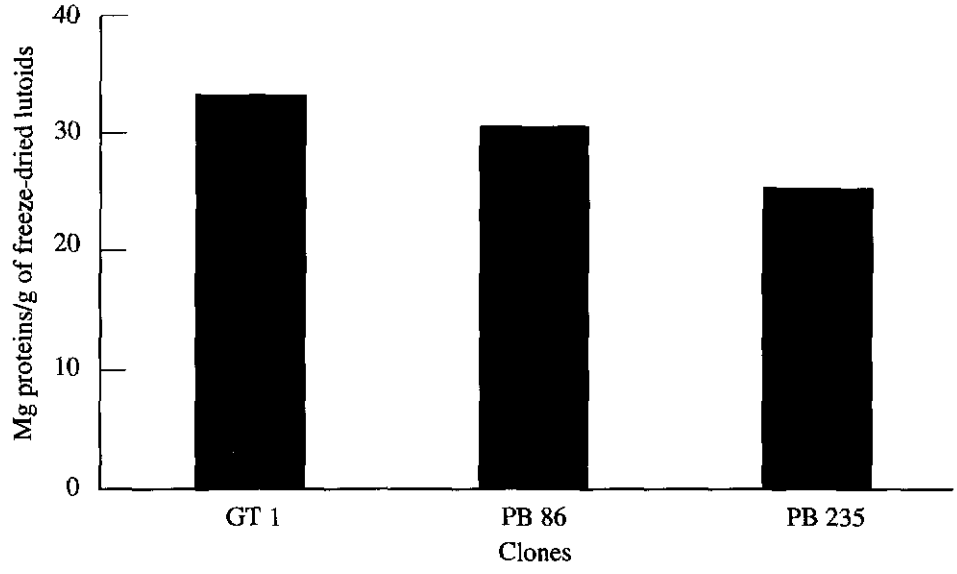

Figure 1. Estimate of the quantity of soluble protein extracted from freeze-dried lutoids from the clones GT 1, PB 86 and PB 235, expressed in mg protein/g of freeze-dried lutoids.

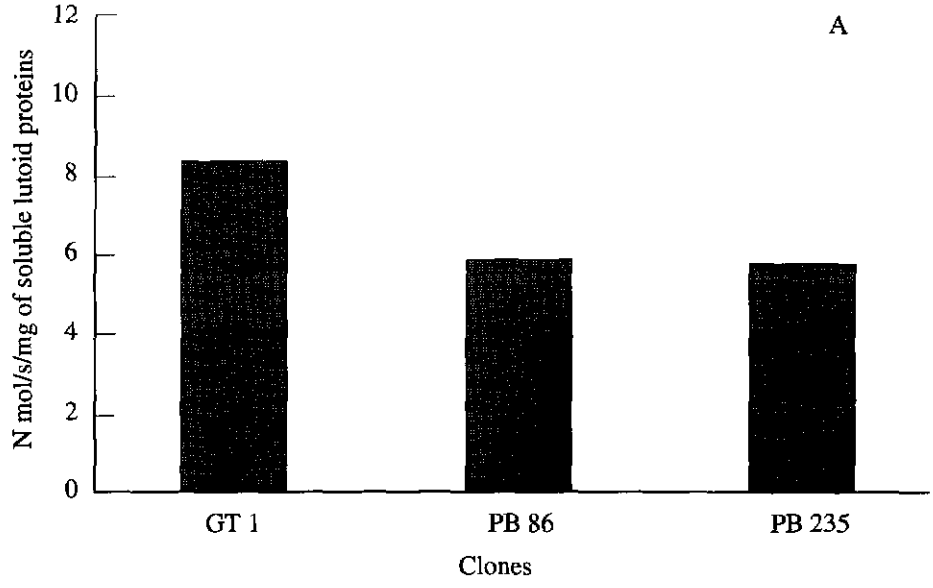

Figure 2. Estimate of lutoid β-1,3-glucanase extracted from clones GT 1, PB 86 and PB 235. Activity is expressed in either n mol/s/mg of soluble lutoid protein (A), or in n mol/s/g of freeze-dried lutoids (B).

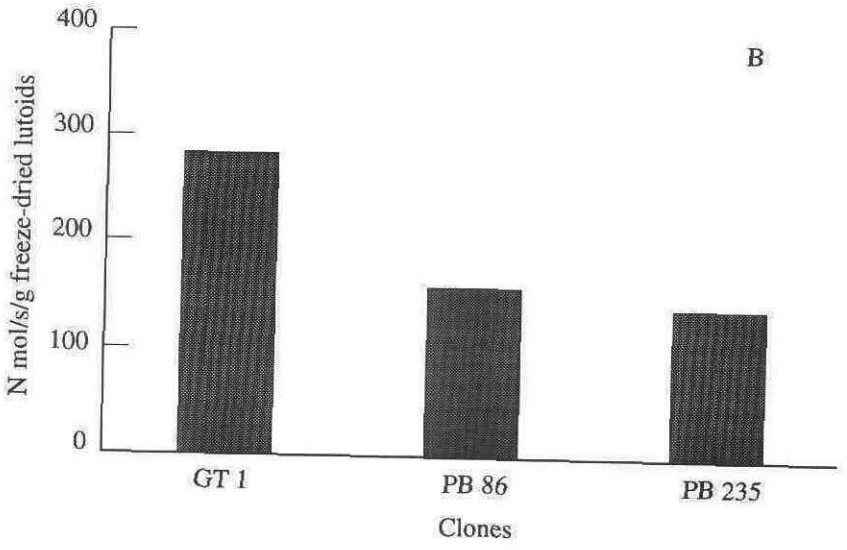

Figure 2 (Contd.). Estimate of lutoid β-1,3-glucanase extracted from clones GT 1, PB 86 and PB 235. Activity is expressed in either n mol/s/mg of soluble lutoid protein (A), or in n mol/s/g of freeze-dried lutoids (B).

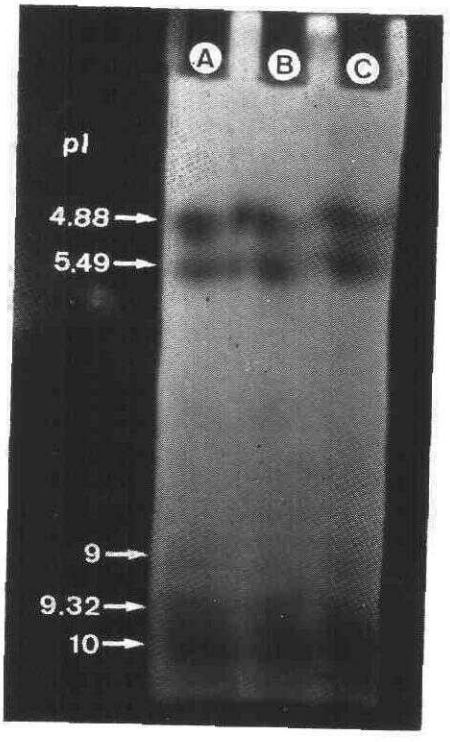

Figure 3. Detection of acid and basic β-1,3-glucanases after isoelectricfocusing under native conditions. The equivalent of 50 µg of soluble lutoid proteins from clones GT 1 (lane A), PB 86 (lane B) and PB 235 (lane C) were deposited in each well.

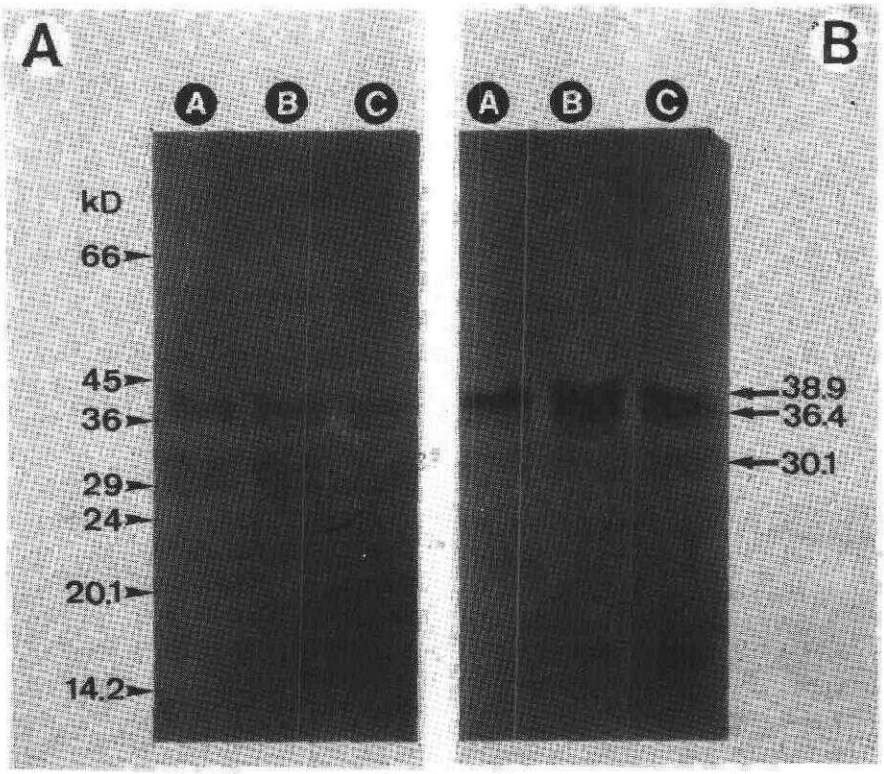

Figure 4. Immunodetection of lutoid β-1,3-glucanases using polyclonal antibodies to acid β-1,3-glucanases (A) and basic β-1,3-glucanases (B) of tobacco. The equivalent of 15 µg of soluble lutoid proteins from clones GT 1 (lane A), PB 86 (lane B) and PB 235 (lane C), were deposited in each well.

tobacco for the same protein group characterised by the same biological function[16,23]. In *Hevea* latex, the two types of antibody display the same protein profile, characterised by three major protein bands with molecular masses of 38.9 ± 1.2 kD, 36.4 ± 0.89 kD and 30.1 ± 1 kD, respectively. It is interesting to compare the co-occurrence of the β-1,3-glucanases and chitinases in lutoids, and their joint elicitation in reactions to plant pathogens, and during abiotic stress. Generally these enzymes are capable *in vitro* of hydrolysing chitin and β-1,3-glucan polymers, which are essential cell wall components of most fungi[27-29]. This synergistic effect enhances the effectiveness of the enzymes in resistance to pathogens[25, 28, 29]. In addition, the exo- and endo-hydrolytic activity of these enzymes, enables them to participate in the release of elicitor oligosaccharides (endogenous and/or exogenous), thus amplifying the defence reactions of the host plant[30,31].

The two enzymes may be present in lutoids, because a producing rubber tree is a system in which stress is continuous through regular wounding by tapping. This must logically cause constant ethylene production[32], and hence a high endogenous level of these enzymes[14]. Ethylene is also one of the messengers

responsible for the elicitation of these hydrolases in reactions of plant to pathogens[26,33] (Breton, unpublished results). The combined presence of these two hydrolytic activities in latex lutoids suggests that they play a role in the resistance of rubber trees to potential pathogens. Without working on latex from a tree tapped for the first time, it is difficult to check whether the two enzymes are constitutive, because latex collection requires physical stress, and so the production of ethylene that may elicit them.

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