Phytochemistry, Vol. 23, No. 4, pp. 747–750, 1984.
Printed in Great Britain.

0031–9422/84 $3.00 + 0.00
Pergamon Press Ltd.

FRACTIONATION OF *HEVEA BRASILIENSIS* LATEX ON FICOLL DENSITY GRADIENTS

FEE-CHON LOW and ANDRES WIEMKEN*

Rubber Research Institute of Malaysia, P.O. Box 150, Kuala Lumpur 01-02, Malaysia; *Laboratory for Plant Physiology, The Swiss Federal Institute of Technology, Zurich, Switzerland

(*Revised received* 6 *September* 1983)

Key Word Index—*Hevea brasiliensis*; Euphorbiaceae; rubber; latex; density gradient centrifugation; ethephon; yield-stimulation.

Abstract—Fractionation of diluted *Hevea brasiliensis* latex on iso-osmotic Ficoll density gradients by sedimentation produces a sharp acid phosphatase-rich lutoid band at the density of *ca* 1.04 g/cm^3. Polyphenol oxidase (PPO) is not exclusively located in the Frey-Wyssling complexes as shown earlier by others; its distribution is variable. A complete separation of acid phosphatase and PPO activities can be obtained by flotation in a Ficoll density gradient. Ethephon stimulation of the tree reduces the acid phosphatase and PPO activities. A transient change in lutoid density soon after stimulation is observed. The occurrence of some new enzyme activities and their subcellular localization are reported.

INTRODUCTION

Density gradient centrifugation of *Hevea* latex was first performed with sucrose as the density medium [1]. With this technique, several lysosomal enzymes were located in organelles known as lutoids (specialised vacuoles in the *Hevea* latex vessels) and polyphenol oxidase (PPO) (EC 1.10.3.1) in Frey–Wyssling complexes (another organelle in *Hevea* latex) [1, 2]. These results were essentially confirmed [3]. The separation of latex organelles in sucrose gradients was however, unsatisfactory because considerable aggregation and flocculation occurred. Sucrose increases the osmolarity considerably along the gradient so that the medium becomes highly hypertonic, causing shrinkage with consequent damage to lutoids. Release of the lutoid contents initiates aggregation of subcellular organelles [4, 5], resulting in lutoids of extremely heterogeneous density upon sucrose density gradient centrifugation [2, 6, 7]. When isotonic Ficoll density gradients were used for the separation of latex organelles, lutoids were found to equilibrate at 1.04 g/cm^3 in a sharp band [8]. The effects of ethephon stimulation on lutoid density had been conflicting [9, 10]. In Ficoll gradients, a distinct second lutoid band with a lower density was found after stimulation.

RESULTS AND DISCUSSION

Fractionation by sedimentation

Centrifugation of diluted RRIM 501 *Hevea* latex on a linear 0–10% Ficoll gradient results in the distribution of marker enzymes as shown (Fig. 1). A narrow band of lutoids as indicated by the high acid phosphatase (EC 3.1.3.2) activity was found around the density of 1.04. Most of the PPO activity was found at the bottom of the gradient whilst a minor but variable portion of the activity was contained in the lutoid band. PPO was reported to be a marker enzyme for Frey–Wyssling (FW) complexes [2].

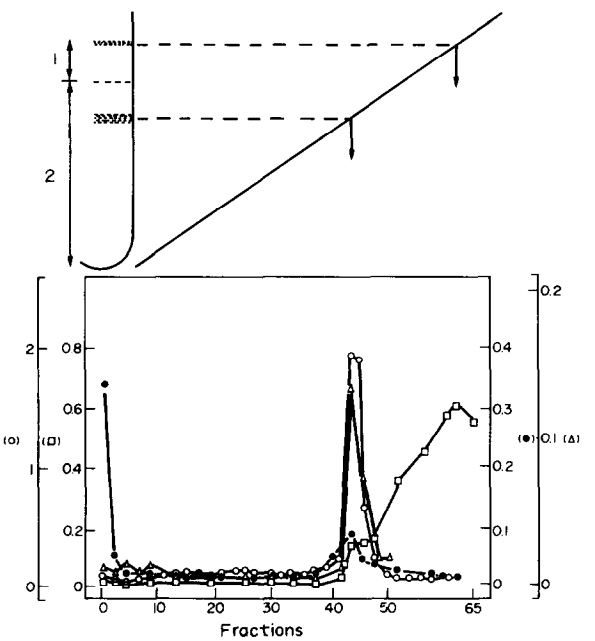

Fig. 1. Separation of RRIM 501 latex on a Ficoll gradient. Diluted latex (1) was loaded on a linear gradient of 0–10% Ficoll (2). After centrifugation in a SW 27 rotor at 10000 *g* for 30 min followed by 70000 *g* for 2 hr, acid phosphatase (○), PPO (●), MDH (□) and diaphorase (△) activities were assayed in the separated fractions (see Experimental).

Hence the finding that the heavy sedimenting PPO activity was not associated with any visible pellet or yellow coloration (indicating the presence of FW complexes) suggests that PPO is not exclusively confined to FW complexes. Similar suggestions have been reported [10,

11] from other evidences.

Diaphorase (a marker enzyme for endoplasmic reticulum) and malate dehydrogenase (MDH) (EC 1.1.1.37) were also found to be associated mainly with the lutoid and soluble (cytosol) fraction respectively. The association of diaphorase with lutoids supports the hypothesis that lutoids are derived from the endoplasmic reticulum (ER) [12].

Fractionation by flotation

Latex made 'heavy' by the addition of 15% Ficoll was fractionated by flotation through a 0–8% linear Ficoll gradient. A typical distribution pattern of the marker enzymes is shown (Fig. 2). Acid phosphatase and α-galactosidase (EC 3.2.1.22), two markers for lutoids were located mainly just above the soluble fraction. A small portion of the activities of these enzymes was contained further up in the gradient, just below the PPO-rich peak. This fraction could contain lutoids at the beginning of differentiation from the ER. Because most of the lutoids are equilibrated just below the soluble fraction, they are least purified by this method. On the contrary, all low specific density organelles, e.g. rubber particles and FW complexes, are purified most effectively, since they float some distance through the gradient. Virtually all the measurable PPO activity was carried to the top of the gradient where a layer of Triton X-100 was present to disrupt floating organelles before they became entangled in the layer of coagulated rubber at the surface of the gradient. By this fractionation, the PPO activity is well separated from the lutoid marker enzyme activities, a separation difficult to achieve with the sedimentation technique [8]. However, it is possible that some of the PPO inhibitor present in the cytosol [11] is carried into the lutoid band. The heavy particulate PPO activity cannot be measured in this gradient because it is not separated from the soluble fraction.

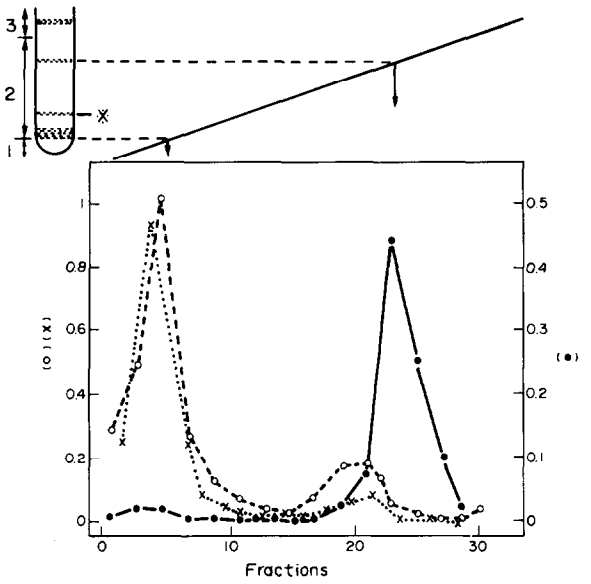

Fig. 2. Fractionation of latex by flotation in a linear Ficoll gradient. The density of diluted latex was increased by the addition of 5 vols of 15% Ficoll in buffered osmoticum (1). A linear gradient of 0–8% Ficoll in buffered osmoticum (2) was then layered on (1). On top of (2), equal vols (0.3 ml) of buffered osmoticum and 1% Triton X-100 in water were layered (3). After centrifugation in a SW 50 rotor at 8000 *g* for 15 min followed by 200000 *g* for 2 hr, acid phosphatase (○), PPO (●) and α-galactosidase (×) activities were assayed in the separated fractions (see Experimental). A band without acid phosphatase or PPO activities was observed (·※·).

Electron microscopy (EM)

EM of the material from the lutoid band obtained by sedimentation showed the lutoids to be less aggregated and less contaminated with rubber particles than was observed in the earlier work with sucrose gradients and mannitol as osmotic stabilizer [Low, F. C. and Gomez, J. B., unpublished results]. A curious feature, not yet understood was that many of the lutoids, while preserving their spherical shape, showed small gaps in the surrounding membrane. The fraction contained an occasional FW complex which might explain the faint PPO activity associated with this fraction.

The material at the bottom of the tubes after sedimentation showed no definable particulate structures. Very occasional osmiophilic particles, possibly fragments of FW complexes and some needle-like crystals of unknown composition were seen.

A possible clonal difference in the particulate PPO

Interestingly, fractionation of latex from another higher yielding clone (RRIM 600), under the same conditions as described in Fig. 1, gave a distinctly different distribution of PPO (Fig. 3). Very little PPO activity was found in the sediment and a much larger proportion was associated with the lutoid fraction. However, the total PPO activity measured was much lower than that of the RRIM 501 latex. The RRIM 501 latex being much more yellow than RRIM 600 latex is supposed to contain more PPO-containing FW complexes. However, this difference is not recognized by measuring PPO activity in the

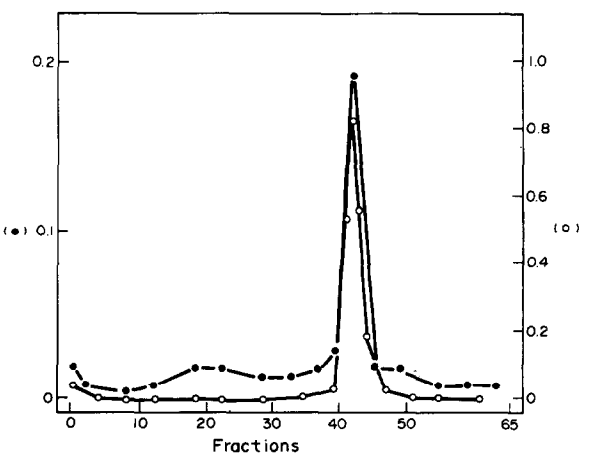

Fig. 3. The distribution of acid phosphatase (○) and PPO (●) activities after the fractionation of RRIM 600 latex on 0–10% linear Ficoll gradient. The gradient was constructed and centrifuged as specified in Fig. 1.

gradients because the FW complexes remain at the top of the gradients, together with the soluble PPO inhibitor, which is probably ascorbic acid.

Effects of ethephon stimulation

Ethephon stimulation of RRIM 600 trees reduced the PPO activity markedly, as measured from the gradients. Acid phosphatase activity was less diminished. A reduced PPO activity after stimulation has been reported [9, 13, 14]. Ethephon stimulation of RRIM 600 trees which had not been stimulated previously demonstrated an interesting feature a few days after stimulation. The single narrow lutoid band regularly seen in latex from unstimulated trees was replaced by two bands. Acid phosphatase and PPO activities were present in these two bands, though their activities were diminished compared to the controls (Fig. 4). The apparent change in lutoid density with stimulation was however, only transient. Lutoid density appeared to be 'normal' again by about the tenth day after stimulation. This transient change in lutoid density with ethephon stimulation has also been observed in RRIM 701 trees which were exploited and stimulated for the first time [15].

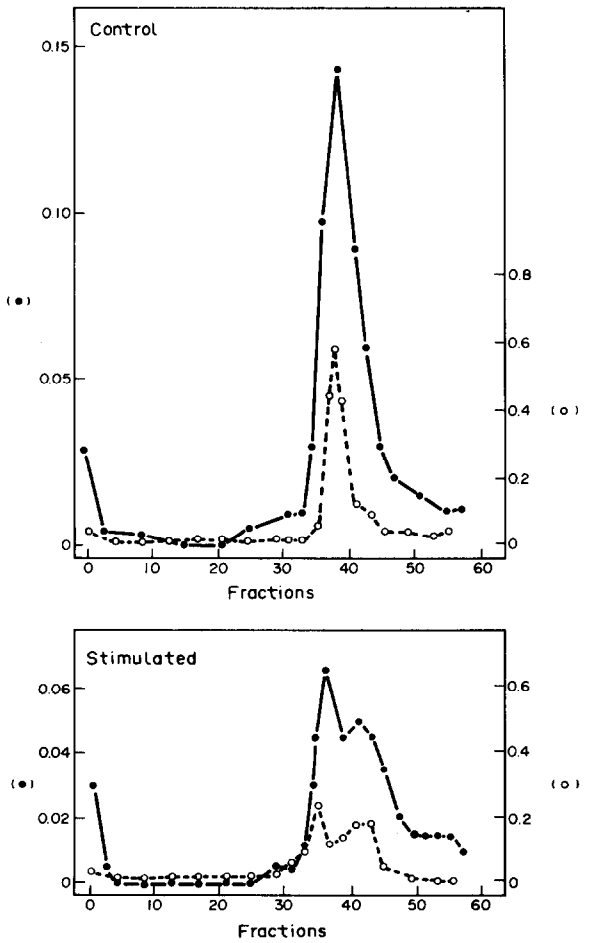

Fig. 4. Comparison of the distribution of acid phosphatase (○) and PPO (●) activities in stimulated and unstimulated RRIM 600 latex after fractionation on 0–10% linear Ficoll gradient. Gradient construction and centrifugation are as described in Fig. 1.

New enzymes

Peptidases and proteases. Both B and C sera (content of lutoids and cytosol, respectively) contained a high activity of leucine aminopeptidase (EC 3.4.1.1) with a pH optimum at 7.6. The activity (per ml) of C serum was twice that of B serum. A weak lysine aminopeptidase was found in both B and C sera. Negligible or no activity could be detected in B and C sera with BANA, BTNA and SUPNA as substrates. B serum had a weak protease activity on Hide powder azure.

Glycosidases. A very active α-galactosidase (EC 3.2.1.22) hydrolysing *p*-nitrophenyl-α-galactopyranoside was detected in the B serum. Since this activity was closely associated with acid phosphatase in the gradients (Fig. 2), it is probably a lutoid enzyme. The pH optimum was 6.0. The pH optima of two other known glycosidases of lutoids, namely, β-galactosidase (EC 3.2.1.23) and β-glucosidase (EC 3.2.1.21) [1, 7] were found to be 4.5 and 6.3 respectively. The relative activities of these three glycosidases measured at their pH optima in the same sample of B serum were β-glucosidase : α-galactosidase : β-galactosidase = 17:16:1.

Oxidoreductases. A very active NADH-dependent dichlorophenolindophenol (DIP) diaphorase was found in the lutoid fraction. The equally active reduction of the redox dye (DIP) observed in C serum was however, non-enzymatic and NADH-independent. This reduction was most probably caused by the ascorbic acid present in this serum [16].

The high activity of particulate DIP-diaphorase makes it a suitable marker enzyme for tracing the ER and ER-derived membranes, like the lutoid membranes within density gradients. This may prove interesting and useful in the study of the origin of lutoids and rubber particles. Other oxidoreductases, e.g. NADH-cytochrome C reductase, present in the lutoids [6, 17] and as markers for the ER, were found to be comparatively much less active.

EXPERIMENTAL

Experimental material. Latex from 15-yr-old RRIM 501 *Hevea* trees was used in all expts, except those on ethephon stimulation, where RRIM 600 latex was used. The RRIM 600 trees (*ca* 15-yr-old) were stimulated for the first time with ethephon as described [18], whilst a similar group of 10 trees remained unstimulated as controls. All the trees were tapped on a half-spiral alternate daily (½S d/2) system in the RRIM Experiment Station, Sungei Buloh, Malaysia. Latex was collected directly into chilled buffered osmoticum 20 min after tapping, to avoid the initial flow fraction which is more unstable and contains more damaged lutoids [19]. The latex, at a final dilution of 1:12 was loaded onto density gradients of Ficoll dissolved in buffered osmoticum. Integrity of lutoids in this diluted latex was routinely checked by measuring the latency of acid phosphatase activity (i.e. difference between total and free acid phosphatase activities, the measurements of which are described below). The buffered osmoticum consisted of HEPES (adjusted to pH 7.9 with KOH) in 0.25 M KCl. This ionic osmoticum was superior to the normally used non-ionic osmoticum (e.g. mannitol) with respect to stabilization of lutoids.

Enzyme assays: free and total acid phosphatase activities (modified from ref. [1]). An aliquot (0.5 ml) of latex, diluted 1:12

with buffered osmoticum was mixed with 1.5 ml 'free' substrate containing 1 mg/ml *p*-nitrophenyl-phosphate in 0.1 M NaOAc buffer, pH 5 and 0.2 M KCl. After incubation at 25° for 10 min, the reaction mixtures were chilled and the reaction stopped by 1 M TCA (2 ml). Colour of the filtrate was developed by mixing 1 M NaOH (2 ml) and 0.2 M Na_2CO_3 (2 ml) with the filtrate (2 ml). The colour was determined at 400 nm.

The estimation of total acid phosphatase activity was essentially similar to that of free acid phosphatase except that 0.5% (w/v) Triton X-100 was included in the substrate. The acid phosphatase activity in the fractions from the gradients was measured with the same substrate-buffer soln as that used for the total acid phosphatase. An enzyme sample (10–50 μl) was incubated at 37° for 15 min. with substrate-buffer (0.5 ml). The reaction was stopped with 0.2 M Na_2CO_3 (1 ml) and the *A* at 400 nm was determined.

Assay for PPO activity (modified from [2]). Enzyme sample (0.05–0.55 ml) was added to DOPA (0.8 ml of 50 mg DOPA in 10 ml 0.1 M Na-Pi buffer, pH 6.1). Buffered osmoticum was used to make up a final vol. of 1.35 ml. The change in *A* at 490 nm was recorded at 20°.

Assay for peptidases and proteases. A number of chromogenic *p*-nitroanilide substrates were tested: Leu NA, Lys NA, BTNA, BANA and SUPNA. Enzyme sample (0.1 ml) was mixed with 0.1 M Tris–HCl buffer, pH 7.5, containing 1% (w/v) mercaptoethanol and 0.5% Triton X-100 (1 ml) and *p*-nitroanilide substrates (0.05 ml at 2 mg/ml in dimethylformamide). After incubation at 37°, the reaction was stopped by boiling (10 min). The change in *A* at 410 nm was recorded.

Hide powder azure protease was estimated by reacting enzyme sample (0.1 ml) with Hide powder azure (0.5 ml at 15 mg in Tris–HCl buffer containing mercaptoethanol and Triton X-100 as described above). After incubation at 37° with vigorous shaking throughout the incubation period, 0.5 M $HClO_4$ (1 ml) was added to stop the reaction. The *A* at 560 nm of the supernatant obtained after centrifugation was measured.

Assay for glycosidases. 0.1 M NaOAc or citrate-Pi buffer (0.4 ml) was mixed with enzyme sample (0.05 ml) and *p*-nitrophenylglycoside substrate (0.2 ml at 2 mg/ml H_2O). Following incubation at 37°, the reaction was stopped by the addition of 0.1 M Na_2CO_3 (2 ml). *A* at 400 nm was determined.

Assay for NADH-DIP-diaphorase. Enzyme sample (0.1 ml) was reacted with a mixture containing 0.1 M Na-Pi buffer, pH 7.4 (0.5 ml); 0.01 M KCN (0.05 ml); DIP (0.05 ml at 29 mg/100 ml); NADH (0.1 ml at 133 mg/ml) and H_2O (0.75 ml). The change in *A* at 600 nm was recorded at 20°.

Assay for MDH. The activity of MDH was measured by the change in *A* at 340 nm in an assay mixture containing enzyme sample (0.1 ml), 0.1 M glycine–NaOH buffer, pH 9.5 (1 ml), 50 mM NAD (0.1 ml), 0.1 M malate (0.1 ml) and H_2O (0.2 ml).

Acknowledgements—The authors wish to thank the Director, Rubber Research Institute of Malaysia for permission to publish this paper. Thanks are also due to Professor G. F. J. Moir for his helpful discussions and to Dr. J. B. Gomez for electron microscopy. The valuable technical assistance provided by the various research assistants in Biochemistry and the preparation of the manuscript by the Plant Science Division office are also gratefully acknowledged.

REFERENCES

1. Pujarniscle, S. (1968) *Physiol. Veg.* **6**, 27.
2. Coupe, M., Pujarniscle, S. and D'Auzac J. (1972) *Physiol. Veg.* **10**, 459.
3. Rubber Research Institute of Malaysia (1977) *Rep. Rubber Res. Inst. Malaya*, 1976, 66.
4. Southorn, W. A. and Edwin, E. E. (1968) *J. Rubber Res. Inst. Malaya* **20**, 187.
5. Southorn, W. A. and Yip, E. (1968) *J. Rubber Res. Inst. Malaya* **20**, 201.
6. Jacob, J.-L., Moreau, F., Dupont, J. and Lance, C. (1976) *Proc. Int. Rubber Conf.* 1975, *Kuala Lumpur.* **2**, 470.
7. Pujarniscle, S. (1971) *Memoire O.R.S.T.O.M. No.* 48, *Paris: O.R.S.T.O.M.* (Office de la Recherche Scientifique et Technique Cutre Mer).
8. Tata, S. J. and Low, F. C. (1979) *Int. Rubber Res. Develop. Board Symp. Kuala Lumpur* 1978.
9. Jacob, J.-L. (1975) *Travaux et Document de L'O.R.S.T.O.M.*, 38.
10. Hanower, P. and Brzozowska, J. (1975) *Travaux et Document de L'O.R.S.T.O.M.*, 25.
11. Brzozowska-Hanower, J., Hanower, P. and Lioret, C. (1978) *Physiol. Veg.* **16**, 231.
12. Gomez, J. B. and Moir, G. F. J. (1979) *Malaysian Rubber Res. Develop. Board Monograph No.* 4, p. 40.
13. Rubber Research Institute of Malaysia (1971) *Rep. Rubber Res. Inst. Malaya*, 1970, p. 47.
14. Rubber Research Institute of Malaysia (1972) *Rep. Rubber Res. Inst. Malaya*, 1971, p. 59.
15. Low, F. C. (1981) Thesis submitted in part fulfilment for the degree D.Sc., University of Gent, Belgium.
16. Archer, B. L. and Audley, B. G. (1973) *Phytochemistry* **2**, 310.
17. Moreau, F., Jacob, J.-L., Dupont, J. and Lance, C. (1975) *Biochim. Biophys. Acta* **396**, 116.
18. Abraham, P. D., P'ng, T. C. and Ng, E. K. (1972) *Proc. Rubber Res. Inst. Malaysia Plrs' Conf. Kuala Lumpur*, 1971, 1.
19. Pakianathan, S. W., Boatman, S. G. and Taysum, D. H. (1966) *J. Rubber Res. Inst. Malaya*, **19**, 259.