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PEROXIDASE FROM *HEVEA BRASILIENSIS* BARK: PURIFICATION AND PROPERTIES

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Key Word Index—*Hevea brasiliensis*; Euphorbiaceae; bark; peroxidase; purification; properties.

Abstract—A peroxidase (EC 1.11.1.7) has been isolated and purified from strips of bark from the rubber tree (*Hevea brasiliensis*). A positive correlation between bark peroxidase level and rubber yield per tapping was observed. High level of peroxidase was found in newly excised bark strips obtained after tapping. The peroxidase converted phenols isolated from the C-serum fraction of centrifuged latex to polyphenolic forms. The peroxidase was purified to homogeneity by size exclusion, ion exchange and affinity chromatography. Gel filtration chromatography and SDS–PAGE indicates that the purified peroxidase is composed of a single polypeptide of M_r 50 000. The enzyme has a pI of 3.5. The K_m values for *o*-dianisidine and H_2O_2 were 20 and 18.6 μM, respectively, and the K_i values for KCN and NaN_3 for these substrates were 10 μM and 2.7 mM, respectively. The possible role of the ethylene-inducible bark peroxidase in latex coagulation is discussed. Copyright © 1996 Elsevier Science Ltd

INTRODUCTION

Peroxidases, which are widely distributed in plants [1], play major roles in the biosynthesis of cell wall polymers [2], are implicated in wound healing [3, 4] and may be involved in auxin catabolism [5] or defence against pathogen attack [6]. Plants contain several peroxidase isoenzymes whose pattern of expression is tissue-specific and developmentally regulated or responsive to environmental stimuli [7]. Wound-induced peroxidases have been studied in potato tubers and root tissues [8, 9].

In rubber production, latex is collected from the rubber tree by a tapping procedure which involves stripping the bark to gain access to the latex stored in the laticiferous vessels of the inner bark. This tapping process induces ethylene production [10]. One of the known consequences of ethylene production is the stimulation of the production of pathogenesis-related proteins, including enzymes. Here we report the characterization of a *Hevea* bark peroxidase, induced by wounding incurred during tapping, and its possible involvement in the regulation of rubber latex flow.

RESULTS

The activity of *Hevea* bark peroxidase (HBP) is positively correlated with rubber latex yield per tapping (Fig. 1). Newly excised bark strips show a very high level of peroxidase activity per unit wet weight compared with that in the latex exudate collected after excising the bark strip by tapping (Fig. 2). The peroxidases in the bark extract, C- and B-sera were separated by PAGE under non-denaturing condition and detected *in situ* by a chromogenic assay. One major and one minor band were found in the bark extract, and two weakly reacting peroxidase components were found in each of the C- and B-sera. One of these components had the same mobility as the major bark peroxidase (Fig. 2). The predominant peroxidase, accounting for >90% of HBP in the bark extract was purified to homogeneity (Table 1) by sequential chromatography on Sephadex G-100 (Fig. 3), DEAE–cellulose and Con A–Sepharose 4B. SDS–PAGE (Fig. 4) and gel filtration showed that the enzyme was a monomeric polypeptide with M_r of 50 000. The pI was determined to be 3.5

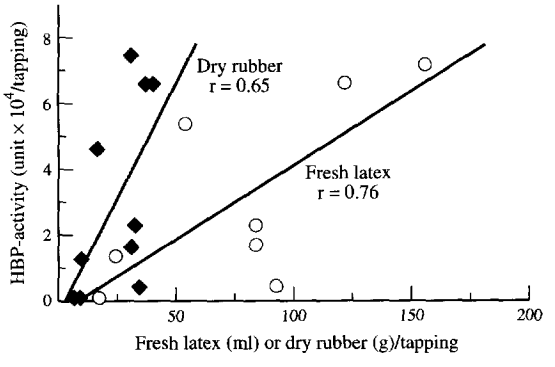

Fig. 1. Correlation between HBP level and rubbber yield per tapping.

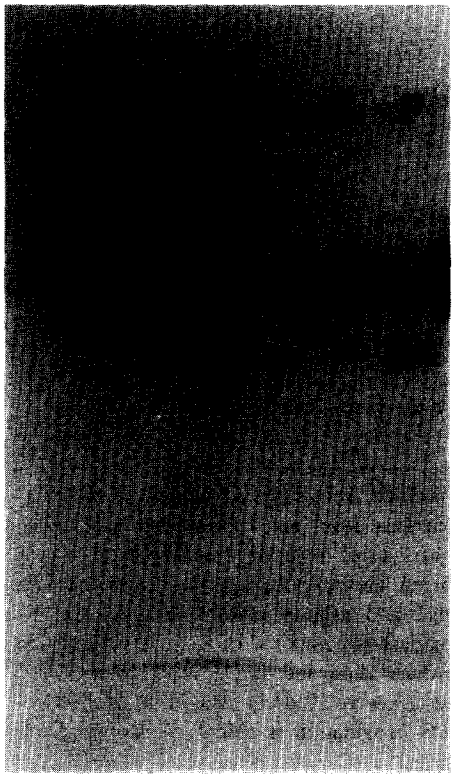

Fig. 2. Peroxidase activity staining on non-denaturing PAGE of samples of equivalent wet wt. A and B, *Hevea* bark strips; C and D, B-serum; and E and F, C-serum.

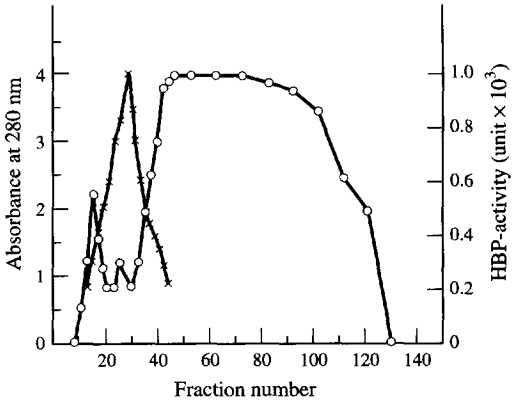

Fig. 3. Elution profile for *Hevea* bark extract on a Sephadex G-100 column.

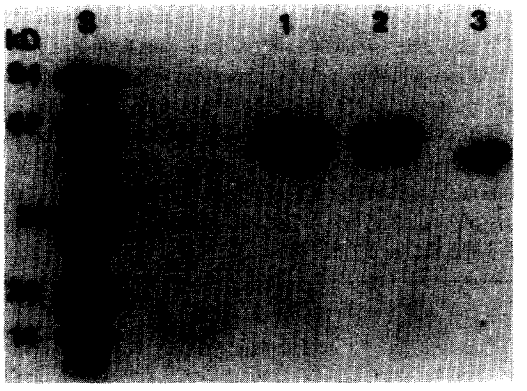

Fig. 4. SDS–PAGE of purified HBP and HRP. Lanes 1 and 2 contained 100 and 50 μg of HBP, lane 3 contained 50 μg of HRP type VI.

(Fig. 5). The K_ms of HBP for *o*-dianisidine and H_2O_2 were 20 and 18.6 μM, respectively. The K_is of HBP in the presence of KCN and NaN_3 were determined to be 10 μM and 27 mM, respectively. When C-serum phenols were used as substrates in the presence of H_2O_2, the reaction products showed an increase in *A* around 260 nm (originally a minimum) and 420–440 nm above the control (Fig. 6).

DISCUSSION

The peroxidase isoenzymes in the bark strips excised during tapping were more abundant than those in the C- and B-sera with a similar PAGE mobility. Other peroxidase isoenzymes different from those in the bark were additionally present in rubber latex. These differences in compartmentalization of the peroxidase isozymes may suggest different *in vivo* functions for the peroxidases in the bark and latex.

The purified HBP consists of a single peptide with M_r of 50 000, similar to that reported for the peroxidase from *Euphorbia characias* latex [11] but higher than that determined for a number of other plant peroxidases [12–14]. The HBP and *E. characias* latex peroxidases have similar K_i values in the presence of KCN and NaN_3.

In latex collection, the rubber tree is wounded every other day by excision of a 1–2 mm thick strip of bark, 0.5–1 cm deep and up to 15 cm long, which results in transverse opening of latex vessels located in the inner

Table 1. Purification protocol of HBP isoenzyme

Fraction	Total protein (mg)	Total activity (u* × 10^5)	Specific activity (u × 10^3/mg)	Yield (%)
Bark extract	1980	30	1.15	100
Sephadex G-100	175	25	14.3	83
DEAE–Cellulose	3.5	7	200	23
Con A–Sepharose 4B	0.9	3.6	397	12

*One unit activity was defined as the amount of enzyme required to produce a change in *A* of 0.1 at 460 nm per min.

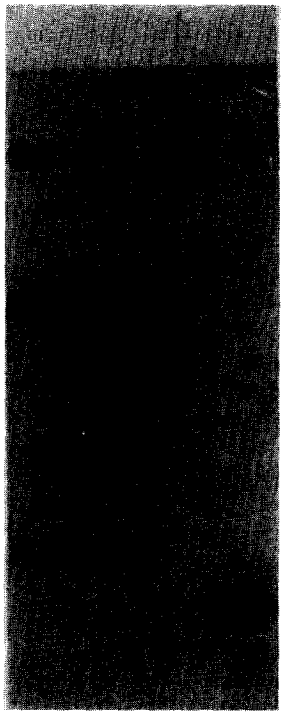

Fig. 5. Isolectric focusing gel electrophoresis of HBP. Lanes S and H represent standard pI marker proteins and HBP, respectively.

bark. Many biochemical changes follow this repetitive injury (tapping) and the ensuing wound healing. One of these biochemical responses is the induction of very high levels of HBP.

The positive correlation noted between HBP level and rubber latex yield suggests that the peroxidase may have an influence on the duration of latex flow. After tapping, latex exudes from the latex vessels in the phloem, which extend some 0.5 m below the tapping site, before coagulation stops the flow. The rubber tree has a mechanism to minimize the loss of metabolic products by inducing formation of a large number of discrete agglomerates, or flocs, composed of degraded latex lutoids (membrane-bound organelles) and rubber particles to form caps which plug the ends of the wounded latex vessels [15]. The duration of latex flow is reported to be dependent on the integrity of the lutoids [16]. The presence of an NAD(P)H oxidoreductase (EC 1.6.99.2) which leads to formation of H_2O_2, superoxide and hydroxyl free radicals has been reported in the lutoids [17]. The release of these free radicals is claimed to be responsible for the peroxidative degradation of lutoid membrane lipids and hence an increase in lutoid membrane fragility [17]. The HBP may have an H_2O_2 scavenging role, as has been suggested for certain bacterial peroxidases [18], with a consequent stabilizing effect on the lutoid membranes, and this would tend to prolong the duration of latex flow in trees with peroxidase-rich bark. Our results also reveal the ability of HBP to convert latex C-serum phenols in the presence of added H_2O_2 to products, tentatively identified as polymerized phenols by the increase in A in the 420–440 nm region, similar to those observed in lignin peroxidase assays [19]. The major change in the 260–280 nm region with the increase in the ratio A_{260} to A_{280} suggests oxidative modification of phenolic compounds [20] probably by the action of peroxidase. An accumulation of phenolic polymers accompanied by an equally rapid and massive increase in the activity of a specific group of anionic peroxidases has been reported in soybean cotyledon tissue following treatment with *Phytophthora megasperma* f. sp. *Glycines* wall glucan [21]. In the rubber tree, the application of ethrel has been shown to lead to a sharp increase in total latex phenols followed by a progressive increase up to 250% [22]. Thus HBP, induced by tapping injury, could convert wound-in-

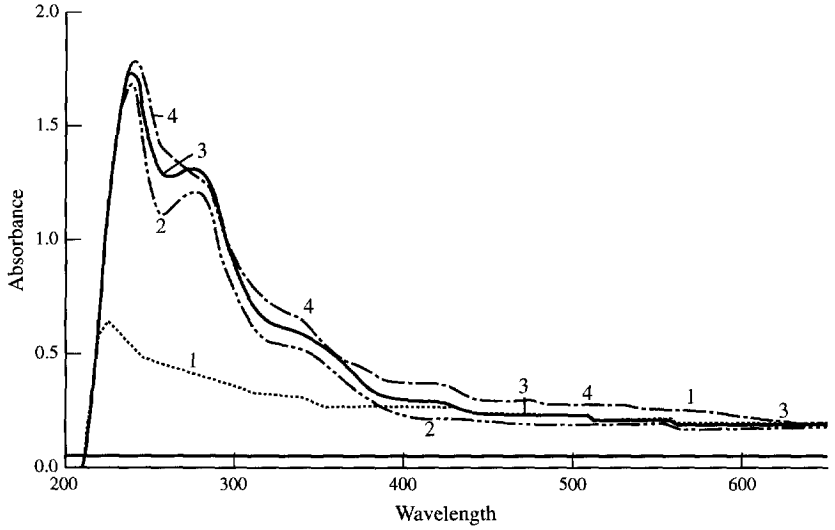

Fig. 6. UV–Visible spectroscopic scans of variously treated C-sera. (1) HBP; (2) C-serum phenol; (3) HBP + C-serum phenol; (4) HBP + C-serum phenol + H_2O_2.

duced latex phenols as substrates into phenolic polymers. These phenolic polymers may enhance latex coagulation due to associations with proteins. Thus the HBP may have opposing effects on latex flow.

EXPERIMENTAL

Chemicals. *o*-Dianisidine, lysozyme, pepsin, ovalbumin, bovine serum albumin, ethrel, DEAE–cellulose, Con A–Sepharose 4B and Sephadex G-100 were from Sigma. H_2O_2 was from Merck. All other chemicals were of reagent grade.

Collection and fractionation of latex. Freshly tapped latex was collected in an ice-chilled beaker from regularly tapped trees of the RRIM 600 clone. The latex was fractionated by centrifugation as in ref. [23] to give a floating rubber fr., C-serum (latex cytosol), and the lutoid (bottom) fr., respectively. B-serum was prepared by repeated freezing and thawing of the lutoid fr. and used as the source of the peroxidase.

Dry rubber yield. Fresh rubber latex obtained after each tapping was oven-dried at 65° to constant wt.

C-Serum phenol preparation. The C-serum fraction (60 ml) was adjusted to pH 12–13 with NaOH, centrifuged (15 000 g for 15 min). The supernatant fr. was sepd and extracted with an equal vol. of $CHCl_3$. The aq. phase containing phenolic compounds was collected, acidified to pH 1 with HC and extracted into $CHCl_3$. The organic fr. was collected, evapd to dryness and the residue dissolved in 5 ml of peroxidase assay buffer.

Bark collection. Newly excised bark strips were collected after tapping, rubber strings were removed and the string-free, bark strips washed with H_2O and used immediately for extraction or peroxidase assay.

Preparation of bark extract. The washed bark (1.8 kg) was homogenized in 10 mM K–P_i buffer, pH 7 (buffer A). The homogenate was filtered through cheese cloth to remove the bark debris and the crude bark extract separated as a clear dark-brown supernatant fr. by centrifugation at 20 000g for 1 hr. The extract was concd by ultrafiltration (Amicon, 10 000 MW cut-off).

Sephadex G-100 chromatography. The concentrated crude bark extract (1 ml) was loaded onto a Sephadex G-100 column (1.8 × 60 cm), previously equilibrated with buffer A and eluted with the same buffer at a flow rate of 20 ml hr^{-1}. Frs (3 ml) were collected and assayed for peroxidase and peak frs pooled.

DEAE–cellulose chromatography. The concd enzyme fr. obtained from Sephadex G-100 chromatography was dialysed and loaded onto a DEAE–cellulose column (2 × 14 cm), previously equilibrated with buffer A at a flow rate of 15 ml hr^{-1}. The column was washed with the same buffer. The enzyme was eluted with buffer A containing 0.1 M NaCl. Fractions (1 ml) were collected and assayed for peroxidase activity.

Concanavalin A–Sepharose 4B affinity chromatography. Peak frs from DEAE–cellulose chromatography were dialysed against buffer A and loaded onto a Con A–Sepharose 4 B column (1 × 5 cm), previously equilibrated with buffer A. The enzyme was eluted with the same buffer containing 0.2 M mannose. Frs (1 ml) were collected for the determination of peroxidase activity.

M_r *determination by Sephadex G-100 gel filtration.* The purified peroxidase obtained after Con A–Sepharose 4 B chromatography was loaded on a Sephadex G-100 column (1 × 90 cm), previously equilibrated with buffer A, at a flow rate of 12 ml hr^{-1}. Frs (1.5 ml) were collected and their *A* at 280 nm and peroxidase activity measured. The column was separately calibrated using lysozyme, pepsin, ovalbumin and bovine serum albumin as M_r markers.

Peroxidase assay. Colorimetic assay. Peroxidase activity was determined by the method of ref. [24]. The assay mixture contained 0.5 ml of 0.05% *o*-dianisidine, 0.1 ml of 0.1 M H_2O_2 in 0.05 M NaOAc buffer, pH 5.4 in a total vol. of 3 ml. Peroxidase activity was measured by the change of *A* at 460 nm at 30° due to *o*-dianisidine oxidation, using the assay mixture, without enzyme, as a blank. One unit of activity is defined as the amount of enzyme required to produce a change in *A* of 0.1 at 460 nm per min.

Spectrophotometric assay. The assay mixture contained 10 μl (4 μg) of purified HBP, 0.5 ml of C-serum phenol, 50 μl of 0.1 M H_2O_2 in 0.05 M NaOAc buffer (pH 5.4) in a total vol. of 0.56 ml. The spectrum of the reaction mixt. was scanned between 200 and 650 nm.

K_m *determination.* Michaelis–Menten constants for the peroxidase substrates *o*-dianisidine and H_2O_2 were determined by incubating 10 μg of purified peroxidase with varying concns of *o*-dianisidine along with fixed saturating concn of H_2O_2 or *vice versa*. The K_m values were determined from double reciprocal plots of enzyme activity and substrate concn.

K_i *determination.* The standard assay mixt. contained 10 μg of purified peroxidase soln with varying concns of *o*-dianisidine and KCN or NaN_3, respectively. The K_i value for each inhibitor was obtained from a Dixon plot in which $1/v$ was plotted against inhibitor concn.

Isoelectric point of the purified HBP (2 μg) was determined by isoelectric focusing on 5% polyacrylamide gel with 2% Biolyte 3/10 ampholytes in Model 111 Mini IEF Cell (Biorad). The potential difference was increased stepwise according to the manufacturer's instructions.

SDS–polyacrylamide gel electrophoresis was performed according to ref. [25]. For non-denaturing PAGE, the same procedure was followed except that the undenatured sample was loaded onto the gel. SDS was removed from the gel by soaking in 20% 2-PrOH soln for 30 min before dipping in peroxidase assay soln for activity staining.

Protein concn was determined by the method of ref. [26].

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REFERENCES

1. Gaspar, T., Penel, C., Thorpe, T. and Greppin, H. (1982) in *Peroxidase 1970–1980, A Survey of Their Biochemical and Physiological Roles in Higher Plants*, p. 1. University of Geneva Press, Geneva, Switzerland.
2. Iiyama, K., Lam, T. B. T., Meikle, P. J., Ng, K., Rhodes, D. J. and Stone, B. A. (1993) in *Forage Cell Wall Structure and Digestibility* (Jung, H. J., Buxton, D., Hatfield, R. and Ralph, J., eds), p. 621. American Society of Agronomy.
3. Espelie, K. E., Franceschi, V. R. and Kolattukudy, P. E. (1986) *Plant Physiol.* **81,** 487.
4. Lagrimini, L. M. and Rothstein, S. (1987) *Plant Physiol.* **84,** 438.
5. Grambow, H. J. and Langen-Schwich, B. (1983) *Planta* **157,** 131.
6. Grisebach, H. (1981) in *The Biochemistry of Plants*, Vol. 7 (Conn, E. E., ed.), p. 457. Academic Press, New York.
7. Lagrimini, L. M., Burkhart, W., Mover, M. and Rothstein, S. (1987) *Proc. Natl Acad. Sci. U.S.A.* **84,** 7542.
8. Imaseki, H., Uchiyama, M. and Uritani, I. (1968) *Agr. Biol. Chem.* **32,** 387.
9. Imaseki, H. (1970) *Plant Physiol.* **46,** 173.
10. Siwei, F., Wannian, Y. and Shaoqiong, Y. (1986) in *Proceedings of the IRRDB Rubber Physiology and Exploitation Meeting* (Yanqing, P. and Canwen, Z., eds), p. 59. Hainan, China.
11. Floris, G., Medda, R. and Rinaldi, A. (1984) *Phytochemistry* **23,** 953.
12. Thomas, R. L. and Jen, J. J. (1980) *Prep. Biochem.* **10,** 581.
13. Decedue, C. J., Rogers, S. J. and Borcher, R. (1984) *Phytochemistry* **23,** 723.
14. Saeki, K., Ishikawa, O., Fukuoku, T., Nakagawa, H., Kai, Y., Kakuno, T., Yamashita, J., Kasai, N. and Horio, T. (1986) *J. Biochem.* **99,** 485.
15. Southern, W. A. (1968) *J. Rubb. Res. Inst. Malaya* **20,** 176.
16. Southern, W. A. and Edwin, E. E. (1968) *J. Rubb. Res. Inst. Malaya* **20,** 187.
17. D'Auzac, J. and Jacob J. L. (1989) *in Physiology of Rubber Tree Latex* (D'Auzac, J., Jacob, J. L. and Chrestin, H., eds), p. 60. CRC Press, Florida.
18. Hochman, A. (1993) in *Plant Peroxidase: Biochemistry and Physiology* (Welinder, K. G., Rasmussen, S. K., Penel, C. and Greppin, H., eds), p. 103. University of Geneva Press, Geneva.
19. Spiker, J. K., Crawford, D. L. and Thiel, E. C. (1992) *Appl. Microbiol. Biotechnol.* **37,** 518.
20. Iiyama, K., Stone, B. A. and Macauley, B. J. (1994) *Appl. Environ. Microbiol.* **60,** 1538.
21. Graham, M. and Graham, T. L. (1991) *Plant Physiol.* **97,** 1445.
22. Coupe, M. and Chrestin, H. (1989) in *Physiology of Rubber Tree Latex* (D'Auzac, J., Jacob, J. L. and Chrestin, H., eds), p. 295. CRC Press, Florida.
23. Wititsuwannakul, R., Wititsuwannakul, D. and Suwanmanee, P. (1990) *Phytochemistry* **29,** 1401.
24. Shannon, L. M., Kay, E. and Lew, J. Y. (1966) *J. Biol. Chem.* **241,** 2166.
25. Laemmli, U. K. (1970) *Nature* **227,** 680.
26. Lowry, O. H., Roseborough, N. J., Farr, A. L. and Randall, R. J. (1951) *J. Biol. Chem.* **193,** 265.