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FINAL REPORT

Covering Period: 15 May 1989-30 June 1993

Submitted to the Office of the Science Advisor
U.S. Agency for the International Development

"MOLECULAR MECHANISM OF YIELD STIMULANT ETHYLENE ON RUBBER PRODUCTION OF *HEVEA BRASILIENSIS*"

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Project Number: 9.347
Grant Number: 493-5542-G-00-9041-00

A.I.D. Grant Project Officer: Mr. Peter H. Deinken

Project Duration: 15 May 1989-30 June 1993

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Executive Summary:

A large variation on rubber yield per tapping per tree is always observed in rubber plantation. The amount of rubber yield from each tapping is dependent on two main factors: a) rubber biosynthetic capacity in tapped latex vessels, functioning in regeneration of new latex in order to fill up the empty vessels due to previous tapping and b) flow capacity for draining the biosynthetic products upon tapping. Therefore, the best way to maximize rubber yield per tapping is to be able to extract latex as close as possible to its biosynthetic capacity of replenishment. Hence, a simultaneous application of both markers on rubber biosynthetic and flow capacities must be employed in selection of high-yield rubber clones.

The marker on rubber biosynthetic capacity was identified to be HMG-CoA reductase , a rate-limiting enzyme in rubber biosynthesis. The project was supported by USAID/PSCT Grant No. 936-5542-G-00-6054-00.

The development of flow marker is more tedious since there are many factors involved in latex vessel plugging process. The fund from USAID/PSTC Grant No. 493-5542-G-00-9041-00 were assigned to cover studies on purification and charaterization of coagulating factors as well as their involvement in the coagulation process. The coagulating factors were identified in rubber bark strip as lectin and in latex membrane-bound organnelle (lutoids) as heat-stable lectin binding proteins. These findings pointed out the importance of lutoid stability in prolonging flow duration. Furthermore, we found the contents of both peroxidase activity in rubber bark strip and phenolic compounds in latex obtained after each tapping to be highly correlated with rubber yield. Studies on protective role of bark peroxidase and latex phenols in stabilizing lutoids in latex, thus leading to an increase in latex stability and increasing its flow time upon tapping is still needed to be further investigated.

In order to persue the study, a full proposal with I.D.# C-14-073, entitled "Markers in Early Selection of High-Yield Rubber Seeding" was recently submitted for USAID/CDR support consideration.

Research Objective:

Rubber latex from *Hevea brasiliensis* is withdrawn from the tree by bark tapping , a thin bark layer (2-4 mm in thickness) is stripped off and latex vessels located mainly in the secondary phloem area will be cut open. Productivity level of tapped rubber is mainly controlled by two major factors; duration of flow after tapping and latex regeneration capacity between two consecutive tappings. After tapping, latex exudes from latex vessels of phloem tissue, most of which is contributed by vessels located near the cut with coveragae over vessels as far as 2 feet below the tapping site. The tree has a mechanism to minimize the loss of metabolites productts by inducing formation of a large number of discrete agglomulates or flocs to plug the wound or proximity ends of latex vessels made by tapping. **The trees with highly efficient mechanisms of wound response give off less latex yield and require less effort to replenish the tapped latex.**

Rubber latex coagulation, both *in vivo* and *in vitro*, begins with the appearance of microflocs of degraded lutoids and rubber particles. It was reported that after tapping, the plugging of lacticiferous vessels will gradually arise and limit itself to the immediate proximity ends of the tapping cut (approximately 1 mm thick of the bark layer below the cut). It is, therefore, conceivable that the formation of microflocs may be induced near the wound and accumulate to form caps which block the latex vessels and stop the flow. Although the involvement of bark and lutoids in latex coagulation has been widely suggested (1-5), neither the identification on lutoidic nor bark coagulating factors has been achieved. Our project objective is, therefore, aimed to identify, isolate, purify and characterize the coagulating factors from *Hevea* bark and lutoid as well as to study the involvement of the coagulating factors in the coagulation process.

Since commercial rubber trees are propagated by grafting, a single tree represents two clones. Therefore, differences in flow duration and biosynthetic capacity reported among trees derived from the same or different clonal grafting types are conceivable (6-9). This study will lead to the development of suitable flow marker for selection and breeding of good flow trees.

Methods and Results:

The coagulating factor in *Hevea* bark was identified as lectin with intrinsic peroxidase activity or so-called POD-lectin. The POD-lectin was purified and characterized as described under **manuscript** I (to be submitted to the Phytochemistry Journal). Another coagulating factor was identified in lutoid as POD-lectin binding proteins with M_r 18 and 22 kDs (see **manuscript** I).

The involvement of coagulating factors in the coagulation process was done by homogenization and extraction of rubber clots formed on the surface of the tapping sites after latex flow cessation (approximately two hours after tapping initation). Binding of POD-lectin with proteins extracted form rubber clot was demonstrated by Westtern blotting of SDS-PAGE obtained from rubber clot protein extract onto nitrocellulose, binding of blotted proteins with POD-lectin and visualizing bound protein by peroxidase activity-staining. The protein content in rubber clot was shown to contain POD-lectin binding proteins with M_r 18 and 22 kDs, the same as those identified to be lutoid coagulating factor (Fig.1). Moreover, antibodies raised against M_r 18 and 22 kDs protein prepared from latex lutoid were able to bind the same POD-lectin binding proteins with M_r 18 and 22 kDs found in rubber clot (Fig.1). These results indicate the involvement of POD-lectin and lutoidic POD-lectin binding protein in the coagulation process.

These findings pointed out the importance of lutoid stability in prolonging flow duration. In additions, we found the contents of both peroxidase activity level in rubber bark strip and phenolic compounds in latex obtained after each tapping to be highly correlated with rubber yield (Fig. 2 and 3, respectively). **Manuscript** II (to be submitted to the Phytochemistry Journal as an adjunct paper with **manuscript** I) described details on difference on peroxidase activity levels per tapping in bark strip and latex obtained from high-, medium- and low-yielding trees as well as methods on purification and characterization of *Hevea* bark peroxidase.

Studies on protective role of bark peroxidase and latex phenols in stabilizing lutoids in latex, thus leading to an increase in latex stability and increasing its flow time upon tapping is still needed.

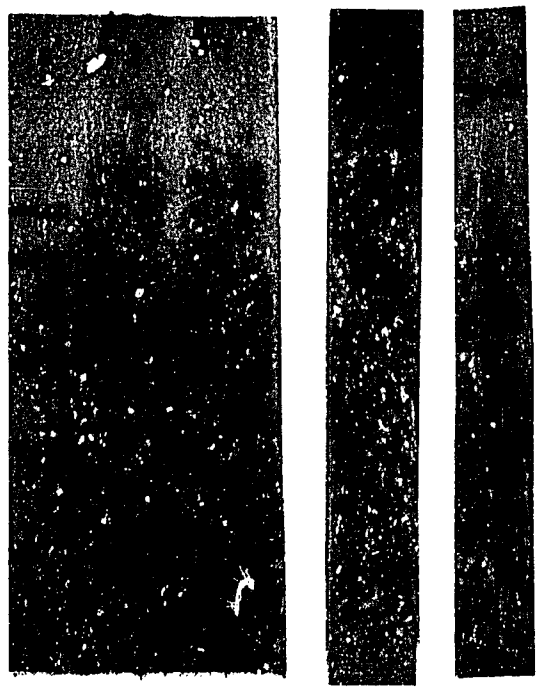

Fig. 1 SDS-PAGE (Coomassie staining) of standard proteins (lane S), proteins extracted from rubber clot (lane 1). The SDS-PAGE of rubber clot proteins (lane 1) was blotted onto nitrocellulose and incubated with either POD-lectin (lane 2) or antibodies against POD-lectin binding proteins (lane 3). The binding proteins were visualized by breif incubation with either peroxidase substrates (lane 2) or anti-rabbit IgG peroxidase conjugate followed by addition of peroxidase substrate (lane 3).

Manuscript I

PEROXIDASE-LECTIN FROM RUBBER (*HEVEA BRASILIENSIS*) BARK

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Key Word Index: *Hevea brasiliensis;* Euphobiaceae; peroxidase, lectin

Abstract:

Lectin was isolated and purified from bark strips of mature rubber (*Hevea brasiliensis)* trees. The M_r determined by SDS-PAGE was 40 kD and that estimated from gel filtration, 140 kD. The protein is able to agglutinate erythrocytes from rabbit and several types of human blood groups but not the erythrocytes from sheep, rat and mice. Proteolytic treatment destroyed all the hemagglutinin activity. The *Hevea* bark lectin was found to possess intrinsic peroxidase activity or so-called peroxidase-lectin (POD-lectin). Similarly, certain types of commercial horseradish peroxidase (HRP) were found to possess hemagglutinin activity. Fetuin, asialo-fetuin, mucin and asialomucin were able to inhibit lectin-induced erythrocyte agglutination of both POD-lectin and HRP-lectin. The kinetic properties of intrinsic peroxidase activity of POD-lectin were different from those found with the purified predominant form of *Hevea* bark peroxidase, which possesses no lectin activity. K_m values for *o*-dianisidine and H_2O_2 were 45 and 43 µM, while the K_i values for KCN and NaN_3 were 21 and 37 µM, respectively. The optimum pH ranged from 4.5 to 9.5. The intrinsic peroxidase activity of POD-lectin revealed to be more heat-stable than those reported for HRP and the predominant form of bark peroxidase having no lectin activity.

POD-lectin exhibited its binding affinity to heat-stable B-serum proteins extracted from the bottom (lutoid) fraction of centrifuged latex. The M_r of lutoidic lectin binding proteins were approximately 18 and 22 kDa.

INTRODUCTION

Plant lectins are a heterogeneous class of proteins or glycoproteins that have in common their unique ability to recognize specific sugar-containing macromolecule (1). Although seeds are the commom source of lectin activity, agglutinations also occur in leaf, stem, latex, roots (1) and bark (2-4). Bark lectins were reported in black-locust (*Robinia pseudoacacia*) (2) , elder (*Sambucus nigera* L.) (3) and *Sophora japonica* trees (4). Recently, a specific class of enzymic hemagglutinin or α-galctosidase-hemagglutinins were repoorted in legume species namely *Pueraria thunbergiana*, *Thermopsis caroliniana*, *Lupinus arboreus*, *Phaseolus limensis* (5), *Vigna radiata* (6,7), *Glycine max* L. (8) and *Vicia faba* (9).

Although at present the role of plant lectins is still unknown, their widespread distribution throughout the plant kingdom suggests their being physiologically important to the plants. A number of roles have been proposed for plant lectins, such as their function in cell wall extension (10), stimulation of cell growth and proliferation (11) and carbohydrate transport (12,13). Following reports on glycosidase activities of legume lectins, the hypothesis for lectin to function as carbohydrate-specific enzyme has received considerable attention. This paper reports on a new nonlegumiinous enzymic lectin from *Hevea* bark.

RESULT AND DISDCUSSION

Lectin was isolated and purified to homogeneity from *Hevea* bark. It is able to agglutinate erythrocytes from rabbit, almost all human blood groups excepted for AB and not those from sheep, rat and mice (Table I). Proteolytic treatment destroys all the hemagglutinin activity (Table II). An aqueous two-phase partition system of the bark extract was used to extract contaminating polyphenolic compounds into PEG-phase. A conventional protein purification procedure was followed after removal of polyphenolic compounds according to the protocol shown in Table III. The chromatography profiles on DEAE-cellulose (Fig.1) and Biogel P-300 (Fig.2) columns indicate that peroxidase activity exactly copurified with a hemagglutination activity. This suggest a presence of a new class of enzymic lectin or so-called POD-lectin. This finding

prompted us to test the presence of lectin activity in crude and purified commercial horseradish peroxidases (HRP). Similarly, we found very high hemagglutinin activity in purified HRP type IX, moderate activity in type II, small activity in type VI, VIII and crude HRP, while no activity was detected in type VII (Table IV). The specific lectin activity of HRP type IX was 6.72×10^3 titer/mg.

Our purified *Hevea* POD-lectin possesses a specific lectin activity of 1.28×10^4 titer/mg and a peroxidase activity of 1.97×10^4 unit/mg (Table III). The specific lectin activity of *Hevea* POD-lectin is higher than that found with HRP-lectin type IX. The peroxidase activity in POD-lectin accounts for only 0.12 % of total bark peroxidase (Table III) and its specific activity is much lower than that reported for the purified predominatant form of bark peroxidase isozyme containing no lectin activity (14). The calibrated native M_r of purified POD-lelctin by gel filtration was approximately 140 kD, (Fig.3). A single protein band with molecular weight of around 40 kD was revealed under SDS-PAGE (Fig. 4). Fetuin, asialofetuin, mucin and asialomucin were able to inhibit both POD-lectin and HRP-lectin induced erythrocyte agglutination, while a wide range of monosugars did not show inhibition (Table IV). The K_m values of peroxidase activity of POD-lectin for o-dianisidine (Fig. 5) and H_2O_2 (Fig. 6) are higher than those found with purified bark peroxidase exhibiting no lectin activity (14), while the K_i values for KCN (Fig. 7) and NaN_3 (Fig. 8) are slightly lower (14). The optimum pH ranged from 4.5 to 9.5 (Fig. 9). The intrinsic peroxidase activity of POD-lectin was revealed more heat-stable than bark peroxidase and HRP (Fig. 10). The different kinetic and physical properties suggest that POD-lectin and predominant bark peroxidase isozyme may not be related.

Strip-wounding of rubber bark by tapping to open up latex vessels in the inner bark area is usually followed by latex lutoid bursting with a subsequent release of its content (B-serum). The lutoid bursting index was reported to be inversely correlated with rubber yield (15,16). Lutoids were suggested as playing an important role in latex-vessel plugging formation and thereby to impede latex flow (15,16,17). We observed a specific binding between purified POD-lectin with heat-stable B-serum proteins prepared from bursted lutoids (Fig. 11). Under SDS-PAGE, the lutoidic lectin binding proteins possess molecular weight of

18, 22 kDs, respectively. Current studies are aimed at seeing of whether the binding of POD-lectin with B-serum proteins functions as part of the initiation of *in vivo*-wounding response for sealing up latex vessels.

EXPERIMENTAL

Chemicals. O-Dianisidine, DEAE-cellulose, Con A-agarose, Sephadex (Sigma), H_2O_2 (Merk); all other chemicals were of reagent grade.

Bark collection. Newly excised bark strips were collected after tapping. Rubber strings were pulled out from the bark strips. The rubber-free bark strips were washed with distilled water and immediately used for peroxidase purification.

Bark extract preparation. Rubber-free bark was homogenized in a Waring blender in the presence of 10 mM potassium phosphate buffer,pH 7.0. The homogenate was filtered through cheese cloth to remove the bark debris. The crude bark extract was separated as a clear dark-brown supernatant fraction after centrifugation at 20,000g for 60 min. The extract was concentrated by ultrafiltration (10,000 MW cut-off). An aqueous two-phase system was used for partition of polyphenolics into the PEG layer. The concentrated aqueous layer containing lectin protein was subjected to further purification.

DEAE-cellulose chromatography. The aqueous phase of the two-phase separation was dialyzed, concentrated by ultrafiltration and loaded onto DEAE-cellulose column (1.4 x15 cm), Following extensive washing with buffer A, the column was eluted with stepwised gradient of 0.4 and 1.0 N NaCl in buffer A, respectively. The high salt peak fractions containing lectin with peroxidase activity were subjected to further purification.

Bio-gel P-300 chromatography. The lectin fractions (second peak) eluted under high salt from DEAE-Cellulose chromatography was pooled, dialyzed, concentrated and loaded on Bio-gel P-300 column (1.4x50 cm), previously equilibrated with 50 mM Tris-HCl, pH 7.4 containing 0.5 M NaCl. The column was eluted with the same buffer at a flow rate of 7.7 ml/hr and 1-ml fractions were collected. For determination of the native molecular weight of POD-lectin, the same column was used with

bovine serum albumin, aldolase and catalase as standard markers.

Hemagglutination assay. Rabbit red blood cells are harvested by centrifugation and washed three times with phosphate buffered saline solution (pH 7.4). The agglutination assay (final volume: 100 ul) was performed at room temperature in U-microtiter plates. Series of 1:2 dilutions of the tested sample solution in a volumn of 50 ul was prepared and followed by addition of 50 µl of a 1.5% suspension erythrocytes. After 1 hour, the titer was recorded as the reciprocal of the lowest dilution which gave detectable agglutination.

Assay of peroxidase activity. The peroxidase activity was determined by using the method of Shannon et. al. (19). The assay mixture contained 0.5 ml of 0.05% *o*-dianisidine, 0.1 ml of 0.1 M H_2O_2 solution in 0.05 M NaOAc buffer pH 5.4 with a ttotal volumn of 3 ml. Peroxidase activity was determined by the change of absorbance at 460 nm at 30 ^oC due to *o*-dianisidine oxidation, using the solution without enzyme as blank.

Dectection of POD-lectin binding protein. Specific binding of purified bark POD-lectin with lutoid coagulating factors in B-serum was demonstrated by Western blotting of SDS-PAGE B-serum proteins onto nirocellulose, binding of blotted protein with POD-lectin and visualizing bound protein by peroxidase activity staining.

Polyacryamide Gel electrophorsis. SDS-PAGE was performed either in the presence or absence of SDS by the method of ref. (20).

Protein determination. Protein concentration was determined by method of ref. (21)

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Table I

Blood type specificity of POD-lectin

Blood type	Hemagglutination (titer)
Rabbit	24
Human type A	6
Human type B	3
Human type AB	0
Human type O	4
Sheep	0
Rat	0
Mice	0

**Titer is defined as the reciprocal of the lowest dilution which gave detectable agglutination.

Table II

Effect of protease on POD-lectin activity

Treatment	Hemagglutination (titer)
Lectin	8
Trypsin	0
Lectin + trypsin	0
Lectin + heated trypsin	8
Protease K	0
Lectin + protease K	0
Lectin + heated protease K	8

Table III

purification protocol of *Hevea* bark POD-lectin

Purification step	Total activity unit*	Total activity titer**	Specific activity u/mg	Specific activity titer/mg	Yield (%)
Bark extract	1.37×10^7	nd	nd	nd	
Aqeous two-phase	7.25×10^6	6.75×10^4	9.17×10^4	8.54×10^2	100
DEAE-cellulose					
Eluted @ 0.4 N NaCl	3.42×10^6	0	3.04×10^5	0	0
Eluted @ 1.0 N NaCl	3.98×10^3	2.91×10^4	4.99×10^2	3.65×10^3	0.09
Biogel P30C	1.93×10^3	1.26×10^4	1.99×10^3	1.30×10^4	0.04

nd =not determined due to interference contaminating phenolics with the assay

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

** Titer was defined as the reciprocal of the lowest dilution which gave detectable agglutination.

Table IV

Hemagglutinin activity of commercial HRP

HRP*	Hemagglutination (titer)	Peroxidase activity	Rz value
crude	4	53 U/mg	0.5
II	16	200 U/mg	2.2
VI	8	300 U/mg	3.2
VII	-	177 U/mg	3.7
VIII	8	125 U/mg	3.2
IX	64	275 U/mg	3.2

* A stock solution of 0.5 mg/ml was used for the hemagglutination assay

Table V

Inhibition of hemagglutinin activity of POD-lectin and HRP-lectin

Inhibitor	POD-lectin	HRP-lectin
1. N-acetylneuraminic acid (mM)	-	6.5 mM
2. Fetuin (mg/ml)	0.156	0.039
3. Asialofetuin (mg/ml)	0.078	0.019
4. Mucin (mg/ml)	0.313	0.078
5. Asialomucin (mg/ml)	1.25	2.50

No inhibition was observed by D-mannose (40 mM), D-mannosamine (40 mM), N-acetyl-B-D mannosamine (30 mM), D-galactosamine (40 mM), N-actyl-D-galactosamine (40 mM) and N-acetylmeuraminic acid (10 mM).

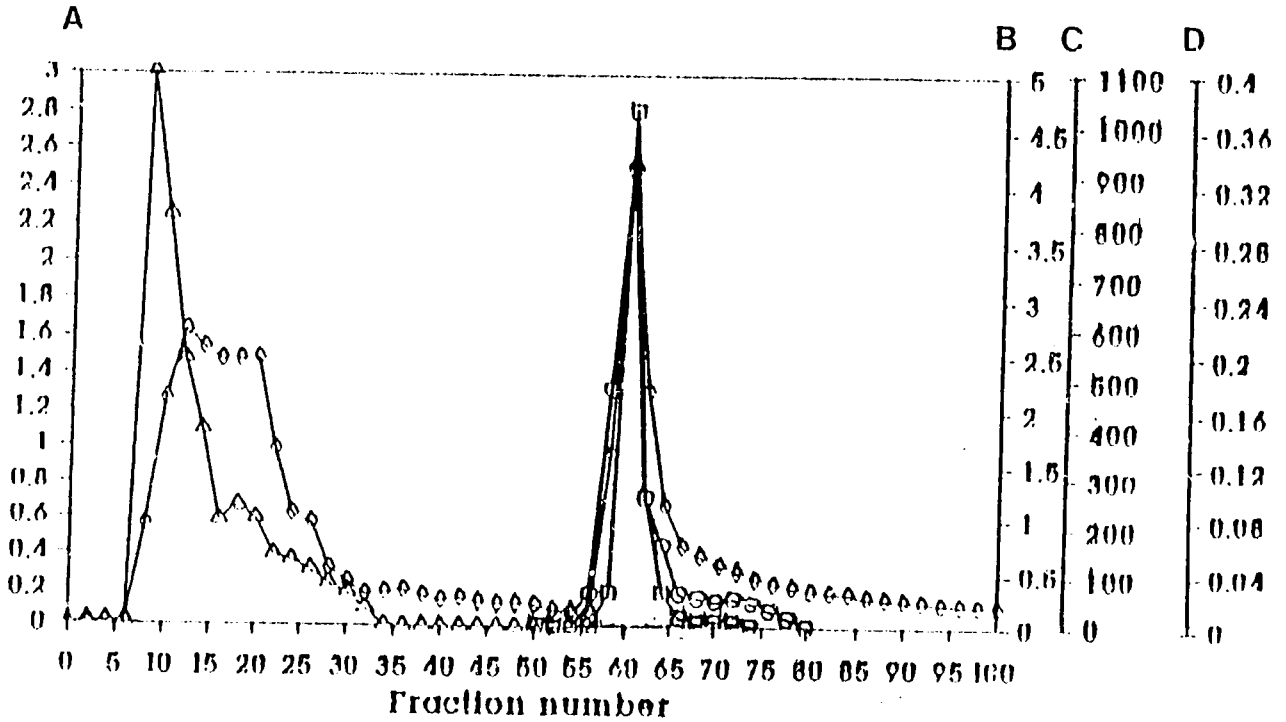


Fig. 1 Chromatographic profile of peroxidase and hemagglutinin activities on DEAE-Cellulose column.
Scale A - OD_{280}, ◊—◊ ; B -hemagglutination (unit), □—□ ;
C and D = peroxidase activity (ΔOD_{460}/ min), △—△ and ○—○ .

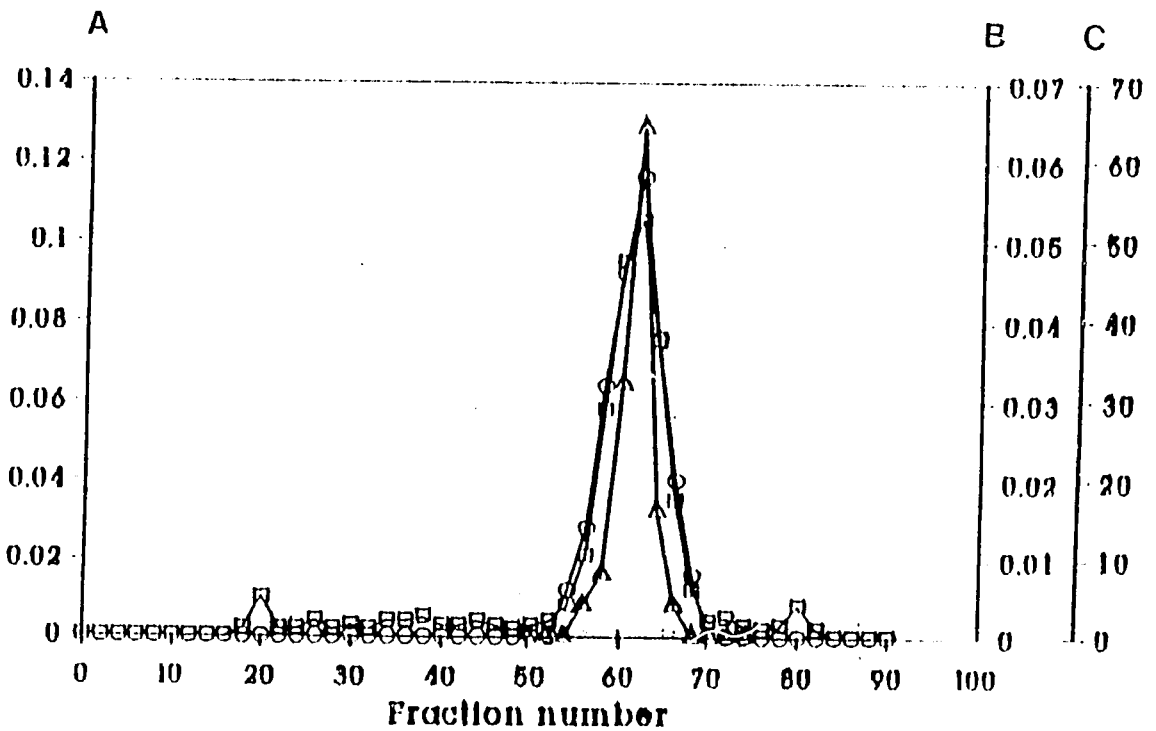


Fig. 2. Purification of active POD-lectin fractions obtained from DEAE-cellulose on Bio-gel P300 column.
Scale A = OD_{280}, □—□ ; B = hemagglutination (unit), △—△ ;
C = peroxidase activity (ΔOD_{460}/ min), ○—○ .

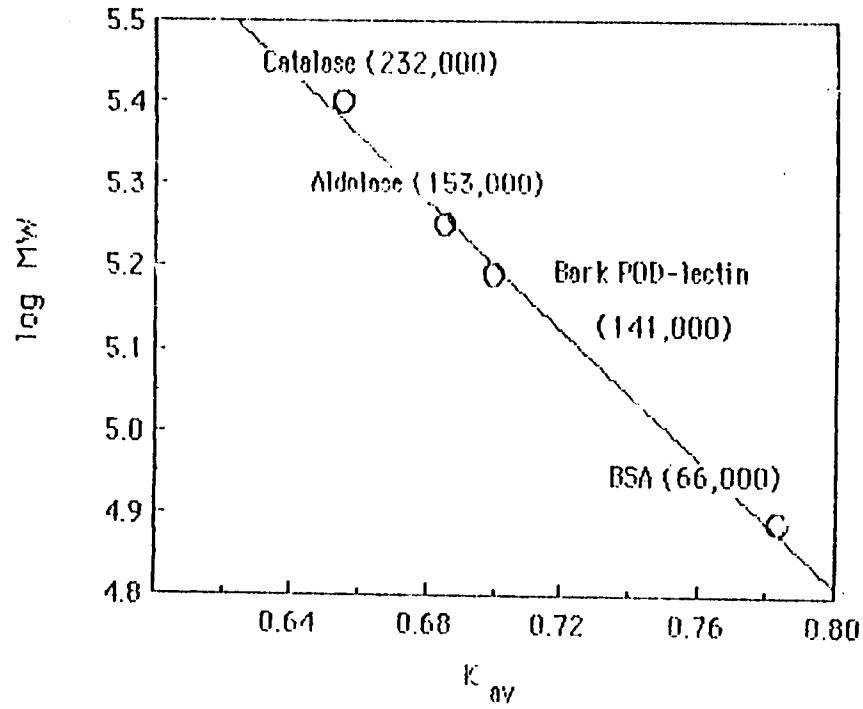


Fig. 3 Relationship between log molecular weight and partition coefficient (K_{av}) of standard proteins and purified POD-lectin in Bio-gel P-300 column.

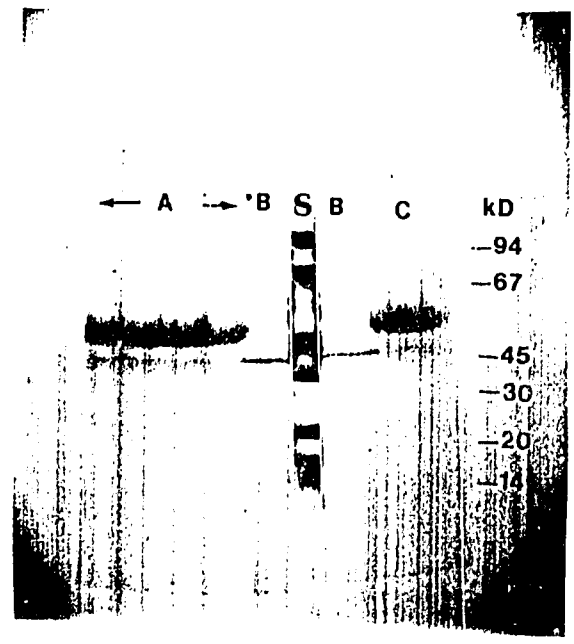


Fig. 4 SDS-PAGE of purified POD-lectin (B) as compared to crude extract (A and C). Lane S represents standard proteins.

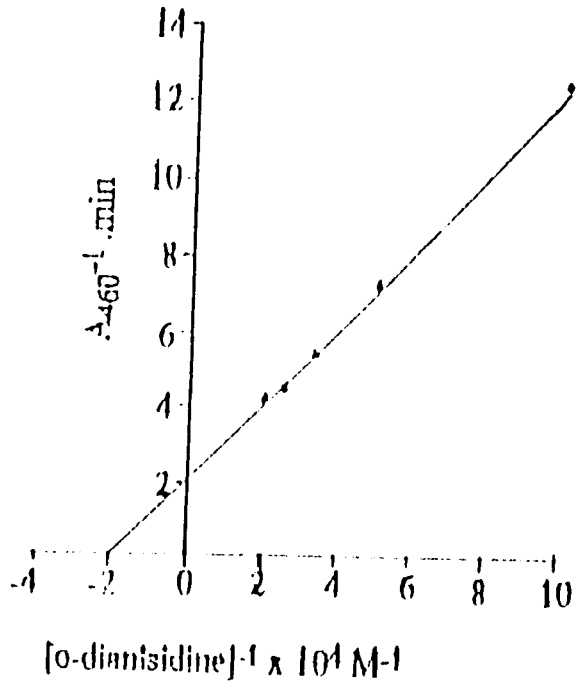


Fig. 5 Determination of apparent K_m for o-dianisidine of intrinsic peroxidase of purified POD-lectin

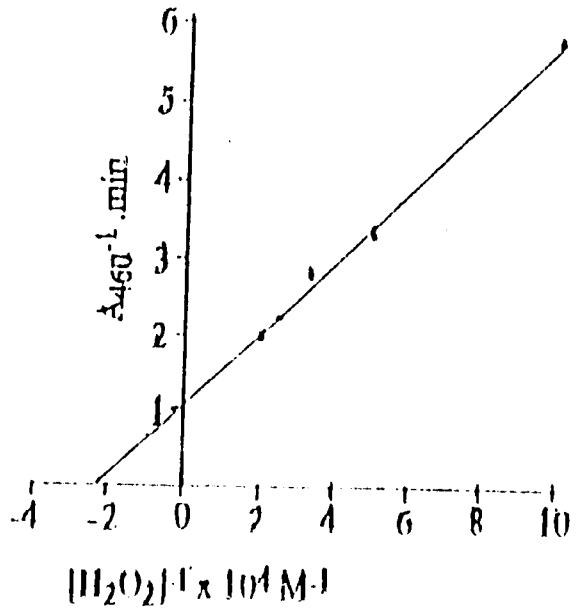


Fig. 6 Determination of apparent K_m for H_2O_2 of intrinsic peroxidase of purified POD-lectin

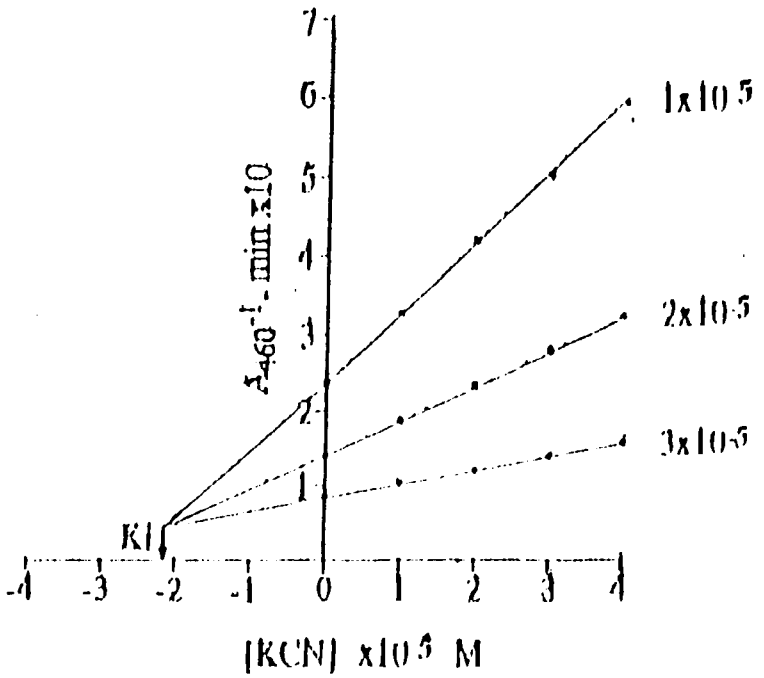


Fig. 7 Determination of K_i for KCN of intrinsic peroxidase of POD-lectin

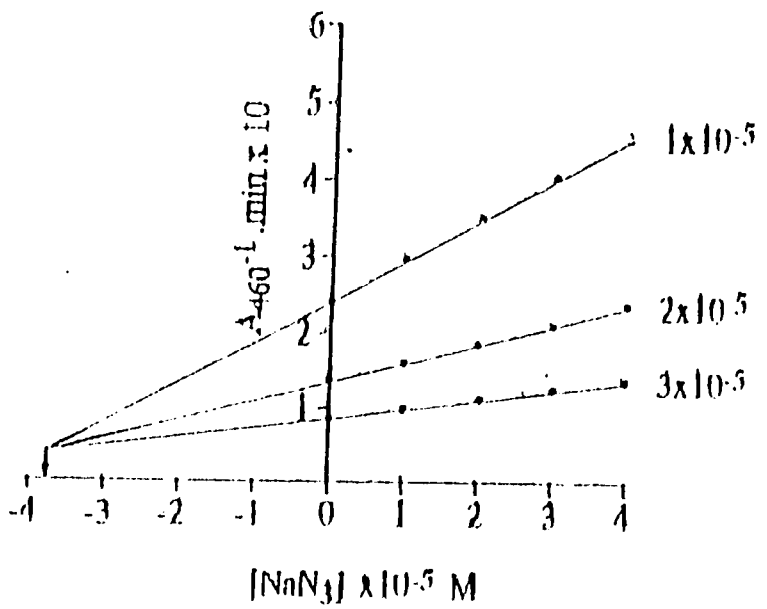


Fig. 8 Determination of K_i for NaN_3 of intrinsic peroxidase of POD-lectin

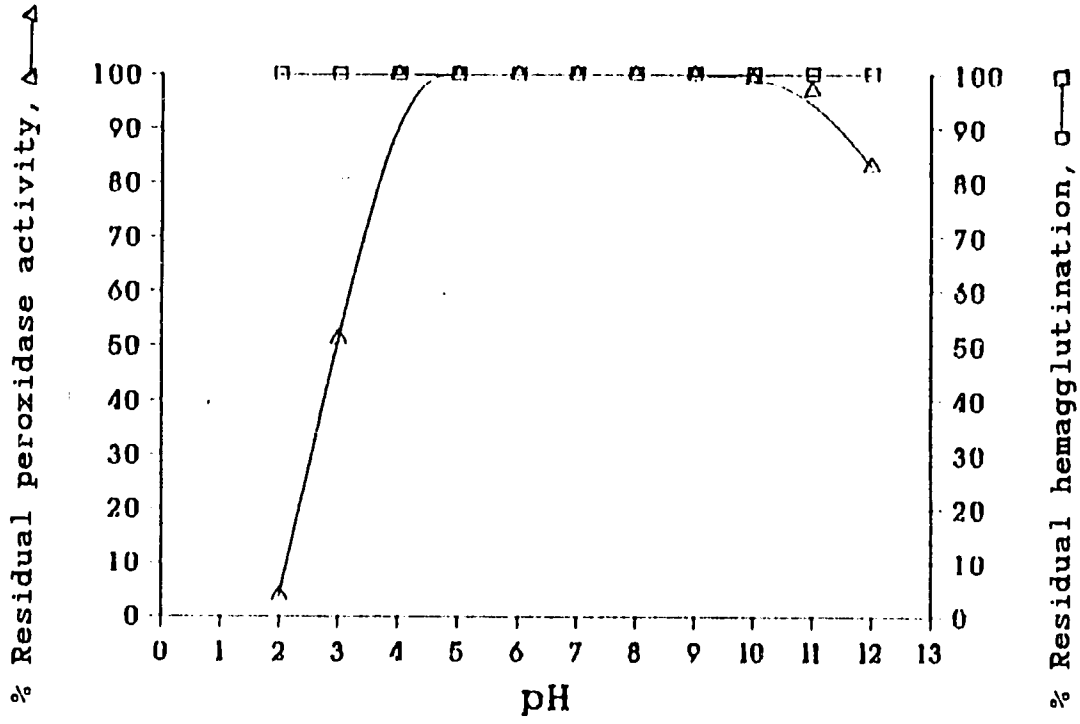


Fig. 9 Effect of pH on hemagglutinin activity and intrinsic peroxidase activity of POD-lectin.

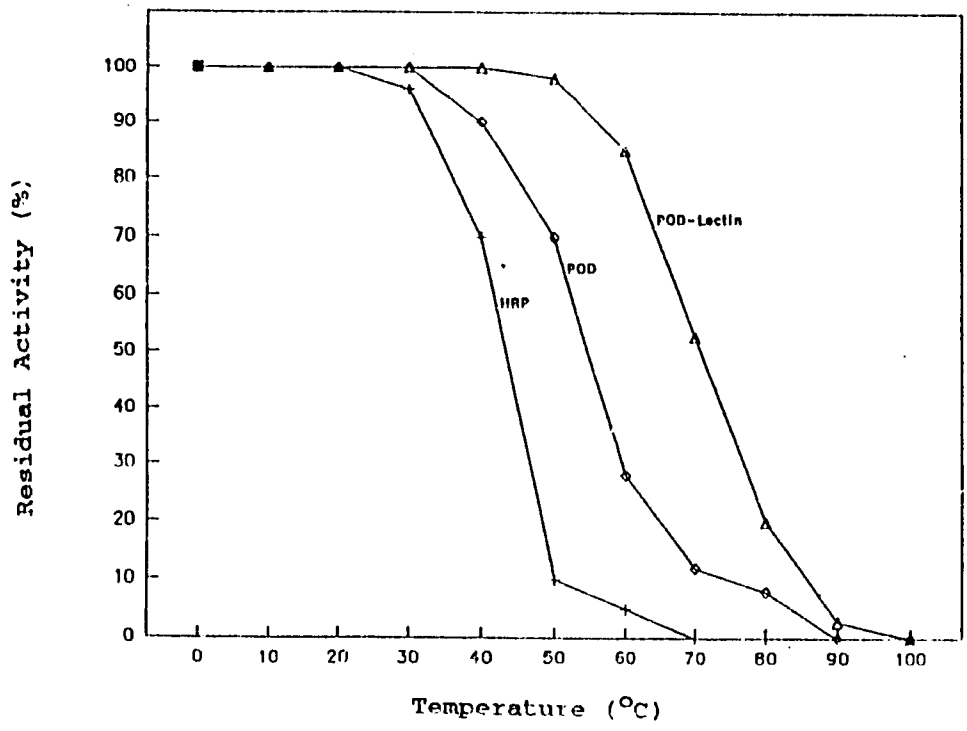
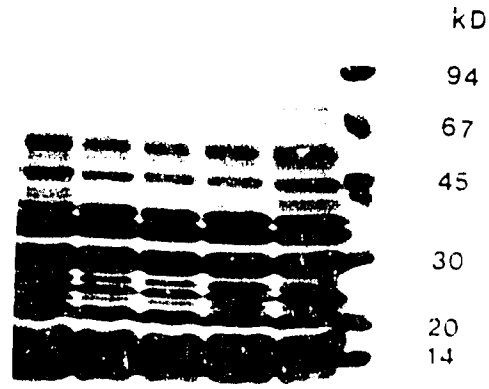


Fig. 10 Heat stability of POD-lectin as compared to *Hevea* bark peroxidase (POD) and HRP.



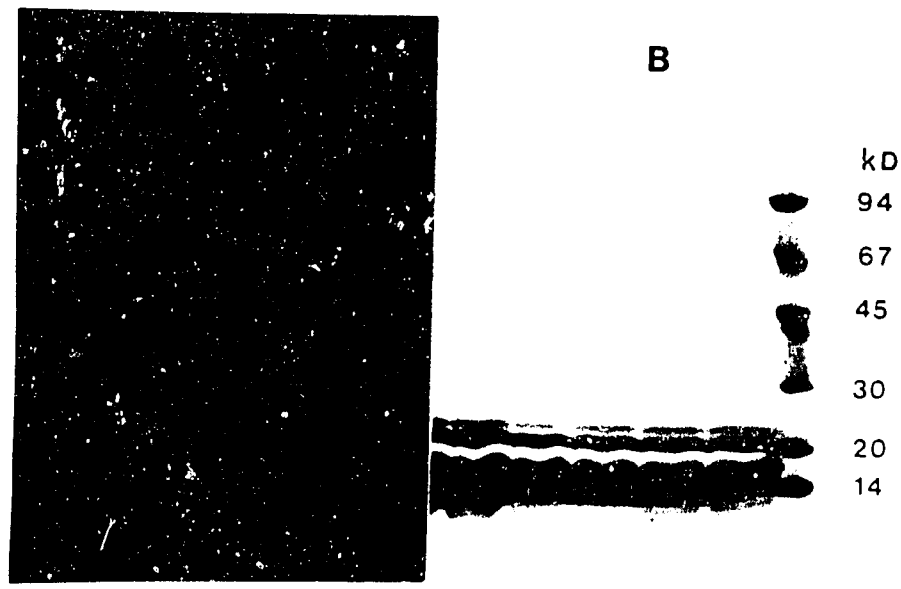


Fig. 11 Specific binding of POD-lectin to 18 and 22 kD proteins of B-serum. A: SDS-PAGE of B-serum; B: boiled B-serum proteins. In parallel, proteins of gel A and B were blotted onto nitrocellulose and incubated with POD-lectin. The POD-lectin binding proteins were visualized by brief incubation with peroxidase substrates (C).

Manuscript II

BARK PEROXIDASE FROM *HEVEA BRASILIENSIS*: PURIFICATION AND PROPERTIES

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Key word Index: *Hevea brasiliensis*, peroxidase, rubber, Euphorbiceae

Abstract:

We have isolated and purified a peroxidase (EC1.11.1.7 donor: H_2O_2 oxidoreductase) isozyme in *Hevea* bark strip. It had much higher activity per unit weight as compared to latex peroxidase either isolated from C-serum or bottom (lutoid) fraction of centrifuged latex. The peroxidase level in newly excised *Hevea* bark strips could be increased by ethylene. Positive correlation between bark peroxidase level and rubber yield per tapping was observed. The bark peroxidase was purified to homogeneity by Sephadex, DEAE, and Con A-Sepharose 4B column chromatography. Gel chromatography and SDS-PAGE indicate that the purified protein is composed of a single polypeptide of M_r 50,000. It is an anionic isozyme with optimum pH of 5.4. It was found to be more heat-stable than horseradish peroxidase. The K_m values for o-dianisidine and H_2O_2 were 20 and 18.6 μM, while the K_i values for KCN and NaN_3 were 34 and 41 μM, respectively.

INTRODUCTION

Peroxidases are widely distributed in the plant and animal kingdoms (1). Plant peroxidases play major roles in the biosynthesis of cell wall and in wound healing, and might be involved in auxin catabloism and defense against pathogen attack (2-5). Plants contain several peroxidase isozymes whose pattern of expression is tissue specific, developmentally regulated and controlled by environmental stimuli (5,6). Cationic

peroxidase isozymes have been reported to be localized in vacuole while the anionic forms were found in cell wall compartment (6,7). Ridge and Osborn (8) reported that in etiolated pea seedlings, ethylene increases both peroxidase activity and hydroxyproline levels in cell walls of the immature region including the apical hook. Later, Stahmann et. al. (9) reported that sweet potato slices infected with the black rot fungus produced low concentration of ethylene and that ethylene *per se* stimulated peroxidase formation, even in uninoculated tissue. Imaseki et. al. (10,11) also showed that the peroxidase content of sweet potato slices was significantly enhanced by a low concentration of ethylene exogeneously supplied to the slices. It was noted by Imaseki et. al. (12) that even though ethylene caused a marked increase in peroxidase activity in sweet potato slices, the ethylene did not alter the composition of isozymes. Kawashima and Uritani (13) showed that the physico-chemical properties of peroxidase isozymes isolated from cut tissue and from tissue infected with black rot fungus were essentially identical.

Tapping injury is performed by stripping off rubber tree bark to collect latex being produced and stored in the inner cortex of lacticiferous vessels. Ethylene is generally known to be induced by machanical wounding including bark injury by tapping (14-16). This report concentrates on the characterization of *Hevea* bark peroxidase, the activity of which is routinely induced by tapping.

RESULT AND DISCUSSION

Newly excised bark strip contains very high level of peroxidase activity as compared to that found for latex peroxidase obtained per tapping. The activity level of bark peroxidase is in the range of 10^4- and 10^5-fold higher than that of C-serum and bottom fraction (B-serum) of centrifuged latex, respectively (Fig. 1). The level of bark peroxidase was correlated with rubber yield. High, medium and low levels of bark peroxidase were found in bark obtained from high-. medium- and low-yielding trees, respectively. On the contrary, the C- and B-sera peroxidase levels were inversely correlated with rubber yield. High-, medium- and low-yielding trees contain low, medium and high levels of C- and B-sera peroxidase (Fig.1 and 2). Activity-staining revealed an intense

color band of bark peroxidase as compared to faint colour observed with the B- and C-sera (Fig.3). The level of bark peroxidase in excised bark strip could be enhanced by simply incubation with fully ripened banana. The best response, giving a 3-fold increase, was observed with the bark strip obtained from high-yielding tree (Fig.1).

Activity-staining of crude bark extract revealed the presence of more than one type of bark peroxidase isozymes (Fig. 3) The most predominant form of isozyme, accounting for > 90% of bark peroxidase, was purified to homogeneity (Table 1) by collumn chromatography on Sephadex G-100 (Fig.4), DEAE-cellulose (Fig. 5) and Con-A Sepharose 4B (Fig. 6). By the aid of SDS-PAGE (Fig. 7), the enzyme was shown to be a monomeric peptide with M_r of 50,000. The same M_r determined by gel filtration (Fig.8). This value is close to that reported for *Euphobia chacias* latex (17) but higher than those reported for other plant peroxidase (18-21). *Hevea* bark peroxidase is apparently an anionic isozyme with pI < 4.5 and has an optimum pH of 5.4 (Fig. 9). It appears to be more heat-stable than horseradish enzyme. A sharp drop from 95 to 10% residual activity of HRP was observed when the temperature was raised from 30-50 ^{o}C, while almost 70% of *Hevea* bark peroxidase still remained active at 50 ^{o}C (Fig. 10). The K_m obtained for *o*-dianisidine (Fig. 11) and H_2O_2 (Fig. 12) were 20 um and 18.6 µm, respectively. The K_i obtained in the presence of KCN (Fig. 13) and NaN_3 (Fig. 14) were 10^{-5} M and $2.7x10^{-3}$ M, respectively. The K_i values obtained were in the same ranges as those reported for *Euphobia chacias* latex (17).

EXPERIMENTAL

Chemical. O-dianisidine, DEAE-cellulose, Con A-Sepharose 4B, Sephadex (Sigma), H_2O_2 (Merk); all other chemicals were of reagent grade.

Preparation of bark extract. Newly excised bark strips were collected after tapping. Rubber strings were pulled out from the bark strips. The rubber-free bark strips were taken washed with distilled water and homogenized in a Waring blendor in the a presence of 10 mM potassium phosphate bufer, pH 7.0. The homogenate was filtered through cheese cloth to remove the bark debris. The crude bark extract was separated as a clear dark-brown supernatant fraction after centrifugation at 20,000 g

for 60 min. The extract was concentrated and subjected to further purification by column chromatoraphy.

Sephadex G-100 column chromatography. The concentrated crude bark extract was loaded onto a Sephadex G-100 column (1.8 x 60 cm), previously equilibrated with 10 mM potassium phosphate buffer pH 7, and eluted with the same buffer at the flow rate of 20 ml/hr. Fractions of 3 ml were collected and assayed for peroxidase activity. Peak activity fractions were pooled, concentrated and further purified on DEAE-cellulose column.

DEAE-cellulose chromatography. The concentrated enzyme fraction obtained after Sephadex G-100 column was dialyzed and loaded onto DEAE-cellulose column (2x14 cm), previously equlibrated with buffer A (10 mM potassium jphosphate buffer, pH 7) at a flow rate of 15 ml/hr. The column was washed with the same buffer A. The enzyme was eluted with buffer A containing 0.2 N NaCl . Fractions of 1 ml were collected and assayed for peroxidase activity. The peak fractions were pocled and subjected to affinify chromatography.

Concanavalin A-Sepharose 4 B affinity chromatography. Peak fractions from DEAE-cellullose chromatography were dialyzed against buffer A and loaded onto a Con A-Sepharose 4 B column (1 x 5 cm), previously equilibrated with buffer A. The column was washed with the same buffer. The enzyme was eluted with buffer A containing 0.2 M NaCl. Fractions of 1 ml were collected and used for the determination of peroxidase activity . The peak fractions were pooled.

Molecular weight determination by Sephadex G-100 gel filtration. The purified peroxidase obtained after Con A-Sepharose 4 B was loaded on Sephadex G-100 column (1x90 cm), previously equilubrated with buffer A , pH 7, at a flow rate of 12 ml/hr. Fractions of 1.5 ml were collected to measure absorbance at 280 nm and peroxidase activity. The same chromatography was run using lysozyme, pepsin, ovalbumin and BSA as standard markers.

Collection and fractionation of latex. The latex was collected from high-, medium- and low- yielding rubber trees of RRIM 600 clones. Latex was fractionated as described in ref. (22). The fractions resulted from latex centrifugation are rubber, C-serum (latex cytosol), and bottom fraction, respectively. The B-serum was prepared by freezing and thawing the bottom fraction. The C- and B-sera were used for measurement of

peroxidase activities.

Assay of peroxidase activity. The peroxidase activity was determined by using method of Shannon et. al. (23). The assay mixture contained 0.5 ml of 0.05% o-dianisidine, 0.1 ml of 0.1 M H_2O_2 solution in 0.05 M NaOAc buffer pH 5.4 with a total volume of 3 ml. Peroxidase activity was determined by the change of obsorbance at 460 nm at 30 ^{o} C due to o-dianisidine oxidation. The incubation mixture without enzyme was used as blank.

K_m determination. Michaelis-Menten constants of peroxidase for o-dianisidine and H_2O_2 were determined by incubating 10 ug of purified peroxidase with varying concentratiions of o-dianisidine as indicated in fixed saturating concentrations of H_2O_2 or *vice versa*. The K_m values were determined from double reciprocal plots of enzyme activity and substrate concentration.

K_i determination. The standard assay mixture contained 10 ug of purified peroxidase solution with varying concentrations of o-dianisidine and KCN or NaN_3, respectively. The K_i value for each inhibitor was obtained from Dixon plot in which 1/v was plotted against inhibitor concentrations.

Polyacryamide gel electrophoresis. PAGE was performed either in the presence or absence of SDS by the method of ref. (24).

Protein determination. protein concentration was determined by method of ref. (25)

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Table I

Purification protocol of major *Hevea* bark peroxidase isozyme

Purification step	Total protein (mg)	Total activity (u)	Specific activity (u/mg)	Purification fold	Yield (%)
Bark extract	1,980	$3x10^6$	1,151	1	100
Sephadex G-100	175	$2.5x10^6$	14,287	9.4	83
DEAE-Cellulose	3.5	$7x10^5$	200,000	131	23
Con A-Sepharose 4B	0.9	$3.6x10^5$	396,640	259	12

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

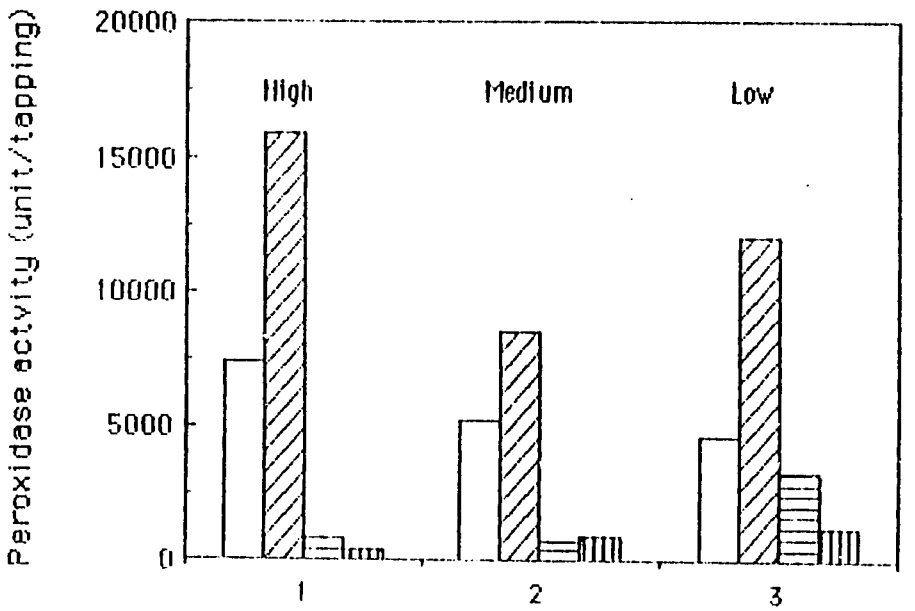

Fig. 1 Peroxidase activity level per tapping was measured in bark strip and latex obtained from high- (group 1), medium- (group 2) and low-yielding (group 3) trees of clone RRIM 600. The total peroxidase activity per tapping was indicated in bark strip as empty bar, C- and B-sera (prepared from centrifuged latex) as bars filled with horizontal and vertical lines, respecively. Hatched bar represents bark peroxidase level after 24 hours incubation with ethylene released from fully ripened banana.

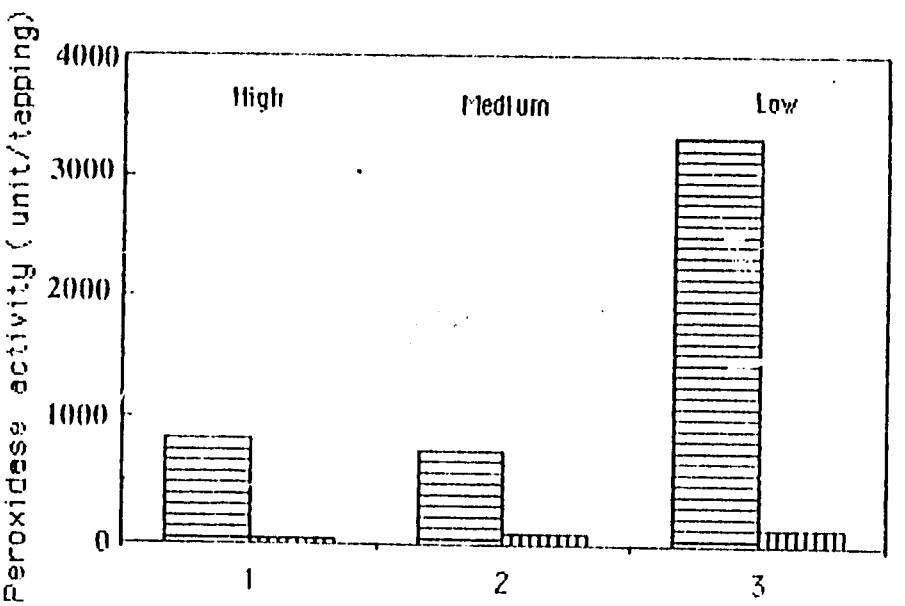

Fig. 2 Peroxidase activity level per tapping was measured in latex obtained from high- (group 1), medium- (group 2) and low-yielding (group 3) trees of clone RRIM 600. The total peroxidase activity per tapping in C- and B- sera were indicated as bars filled with horizontal and vertical lines, respecively.

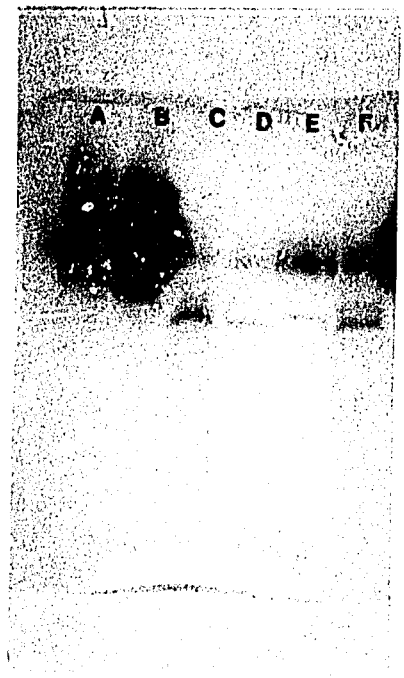

Fig. 3 Activity staining of peroxidase/ unit wet weight of *Hevea* bark (A,B); B-serum (C,D) and C-serum (E,F) obtained from PAGE.

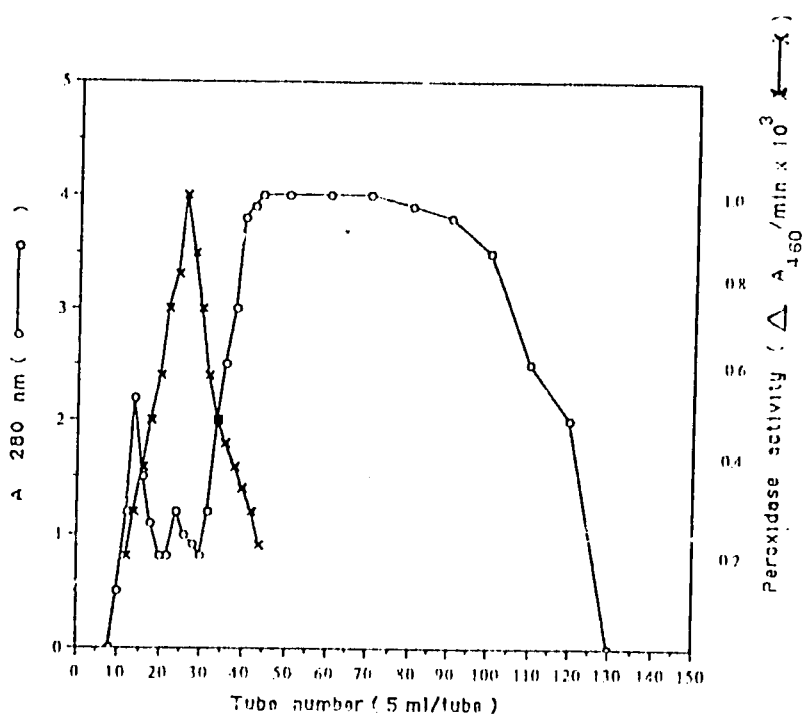

Fig. 4 Chromatographic profile of *Hevea* bark extract on Sephadex G-100 column

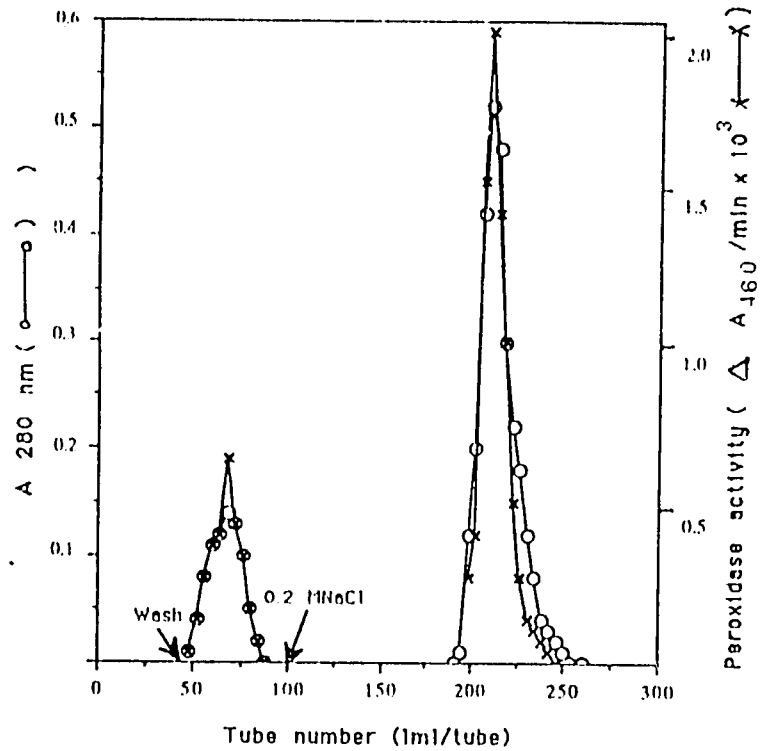

Fig. 5. Purification of active fractions obtained from the Sephadex column on DEAE-Cellulose column.

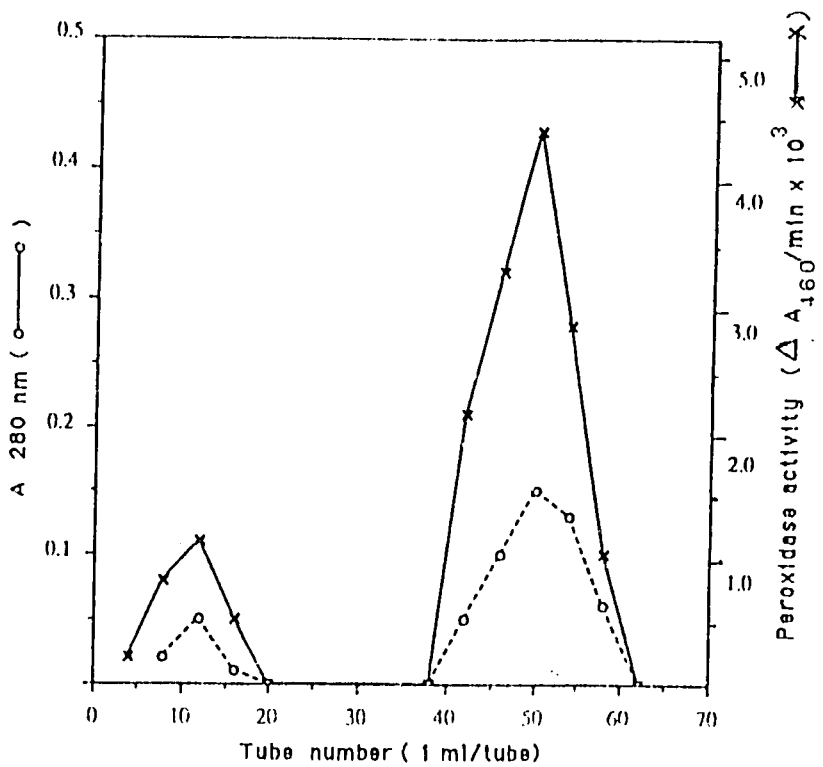

Fig. 6 Chromatography on Con A- Sepharose 4 B column of the active fraction from the DEAE-Cellulose column.

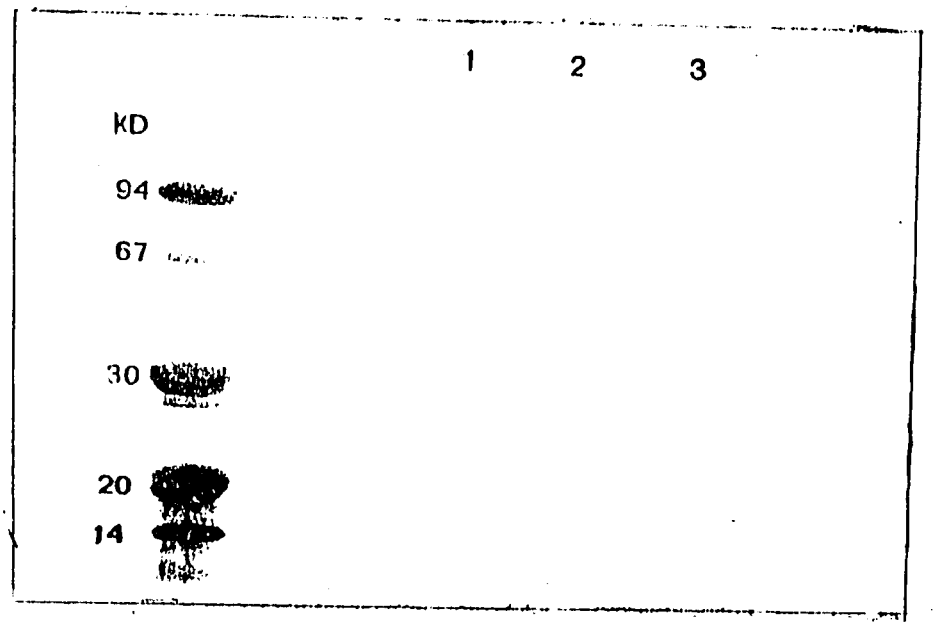

Fig. 7 SDS-PAGE of purified *Hevea* bark peroxidase (POD) and HRP. Lanes 1 and 2 contained 100 and 50 ug of bark peroxidase while lane 3 contained 50 ug of HRP

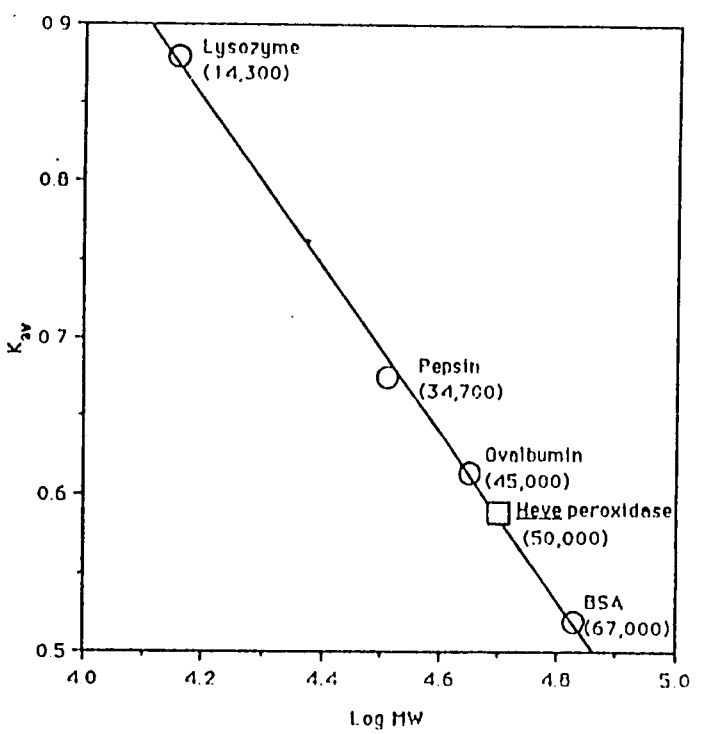

Fig. 8 Relationship between log molecular weight and partition coefficient (K_{av}) of standard proteins and purified bark peroxidase in Sephadex G-100 column

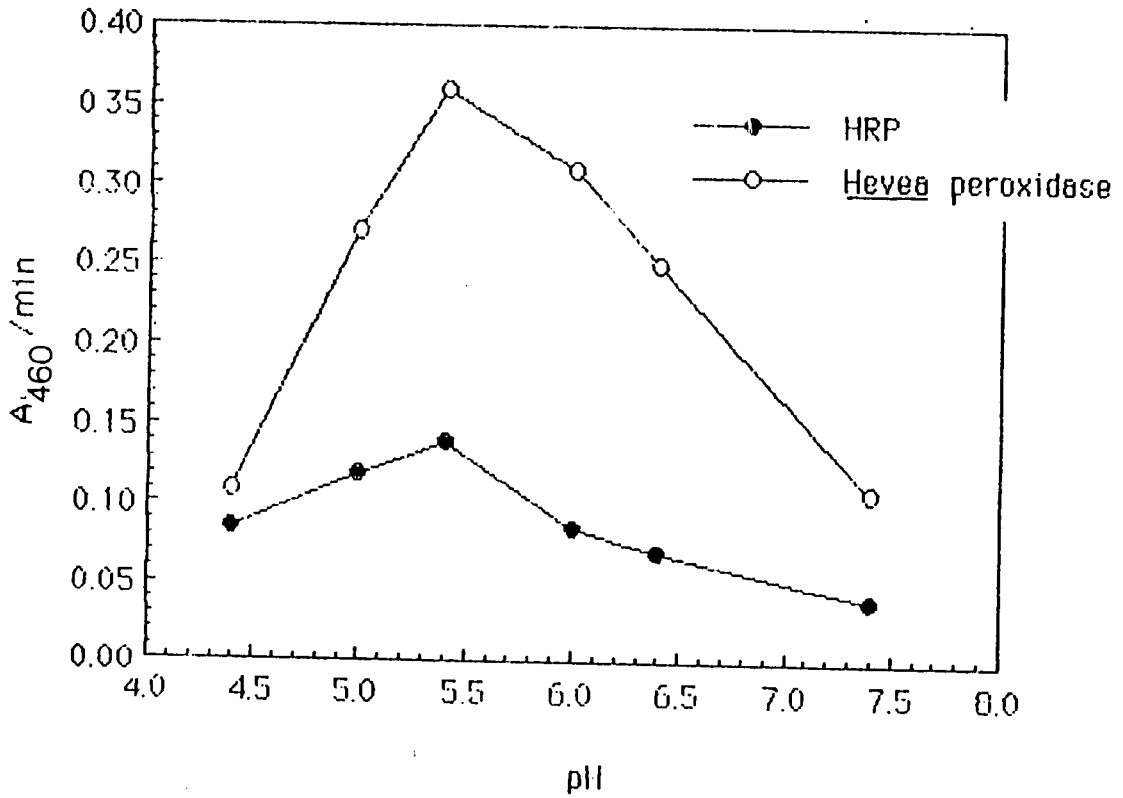

Fig. 9 Effect of pH on *Hevea* bark peroxidase as compared to HRP.

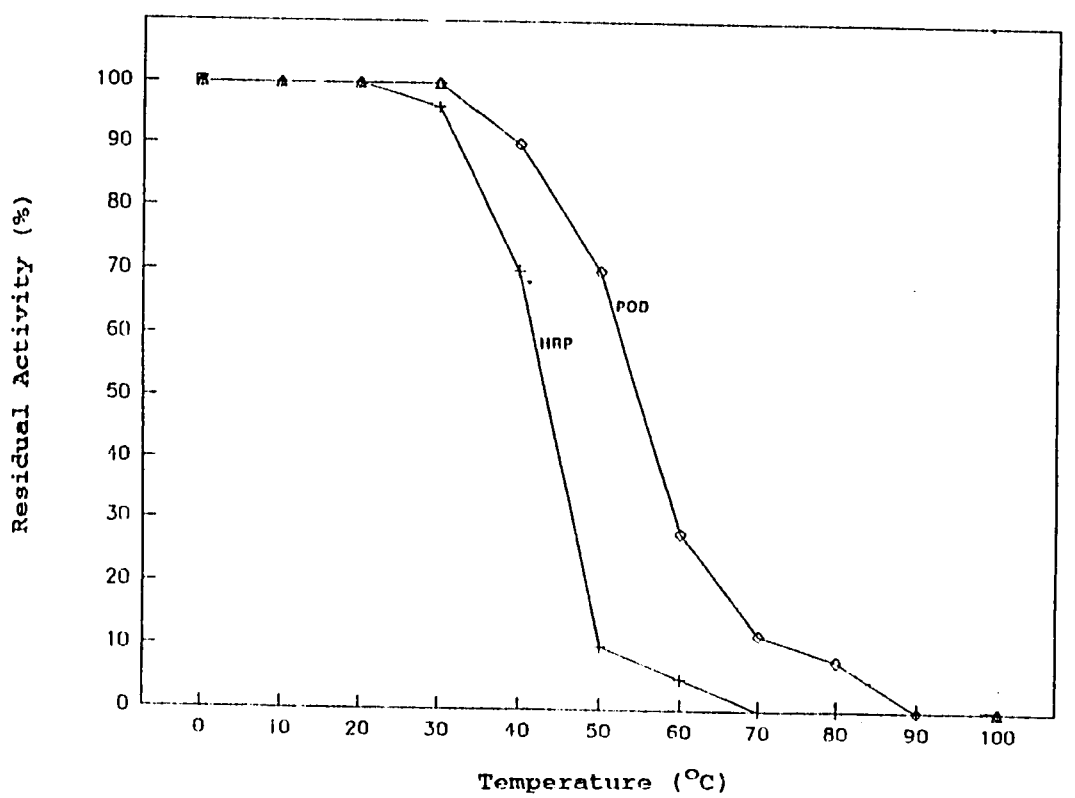

Fig. 10 Heat stability of *Hevea* bark peroxidase (POD) as compared to HRP

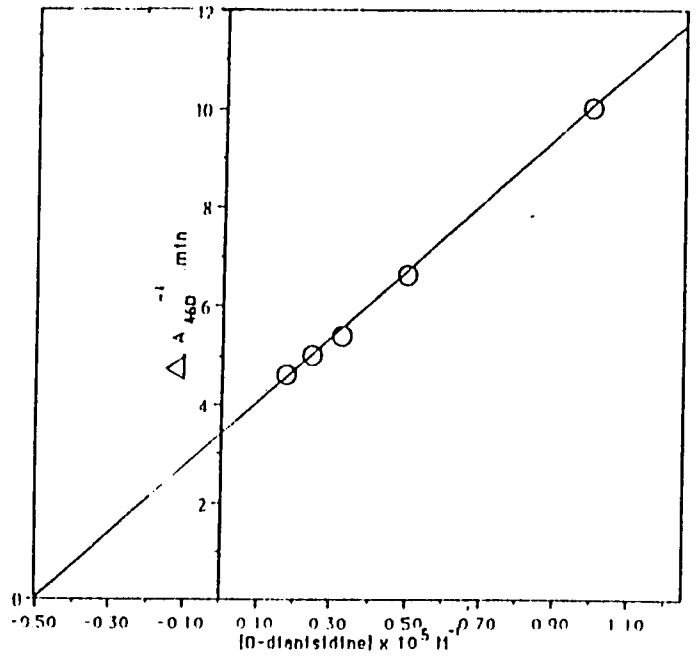

Fig. 11 Determination of apparent K_m for o-dianisidine of *Hevea* bark peroxidase.

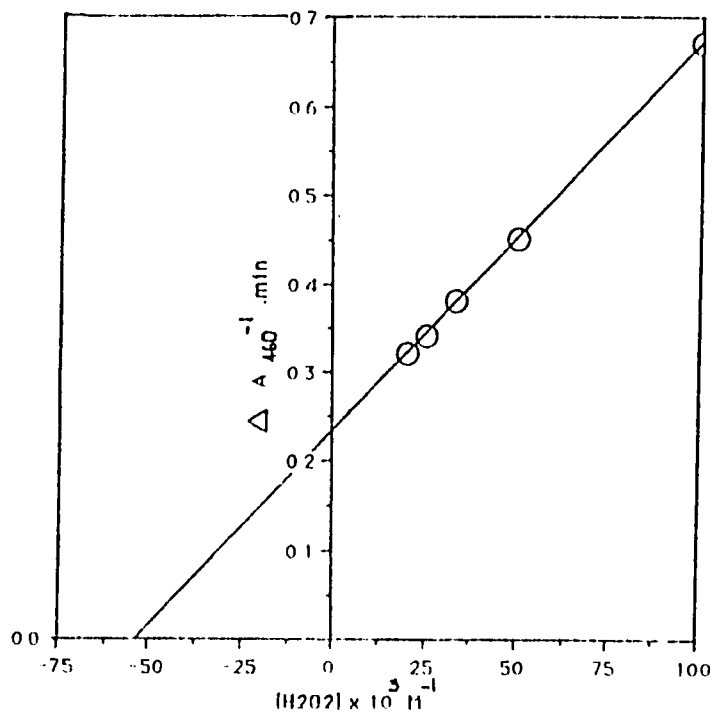

Fig. 12 Determination of apparent K_m for H_2O_2 of *Hevea* bark peroxidase

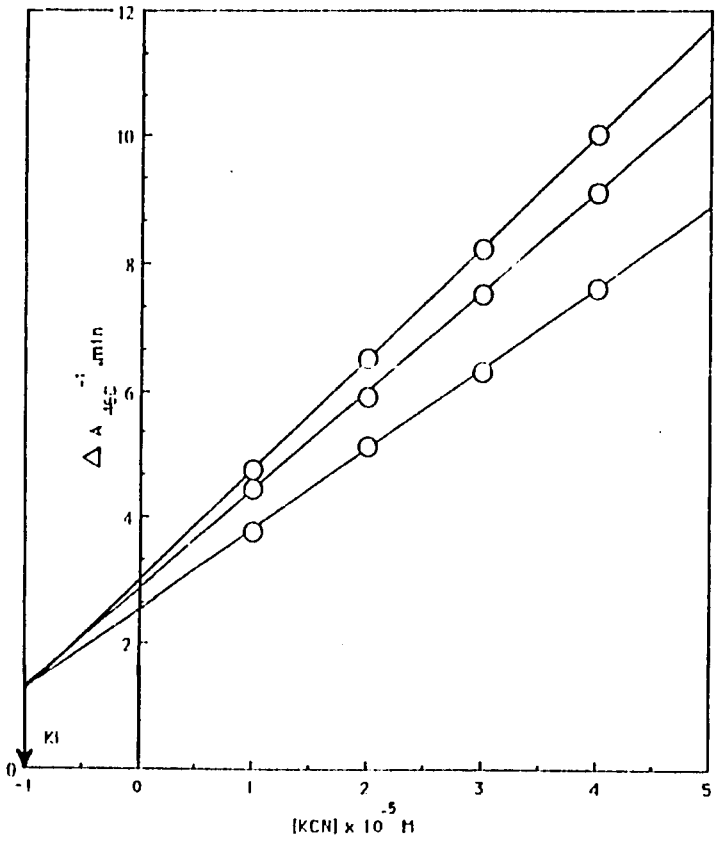

Fig. 13 Determination of K_i for KCN of *Hevea* bark peroxidase

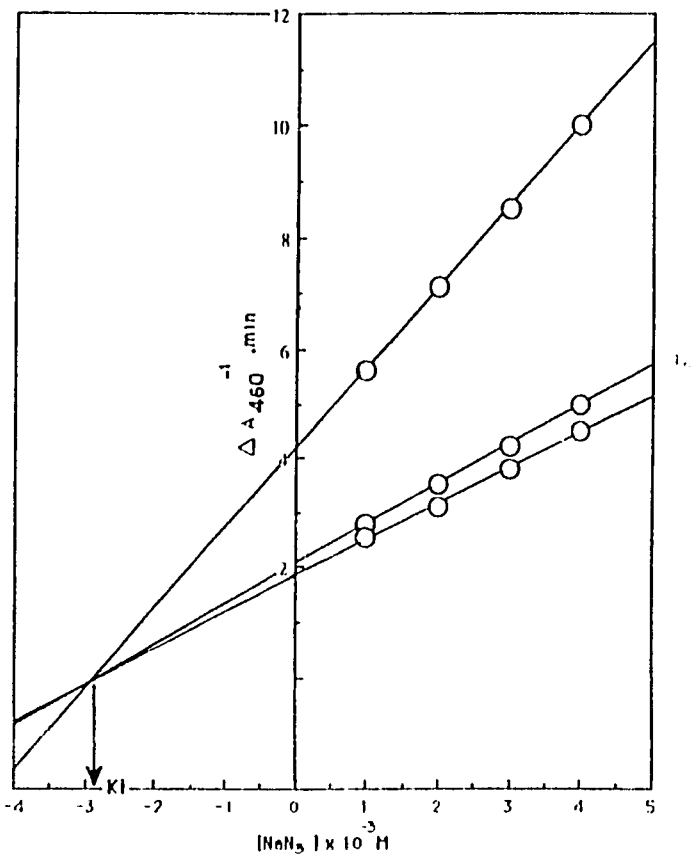

Fig. 14 Determination of K_i for NaN_3 of *Hevea* bark peroxidase

Impact, Relevance and Technology Transfer:

The final outcome of our work will have enormous potential for benefiting the 800,000 rubber farmers in this country, and millions more in elsewhere (i.e. Indonesia, Phillipines, Malasia, Sri Lanka and Brazil etc.). As long as it is economical sustainable to stay in rubber plantation, simultaneous benefit on reforestration program for land conservation can also be achieved. This will help improve global environment.

Throughout the project, we were able to train 3 graduate students, produce three mastes thesises and a few expected international publications (see manuscripts I & II), improve lab frcilities with new equipments on compltete set of Biorad electrophoresis & blotting apparatus and Savant speedvac system.

We have now shown the involvement of bark lectin and lutoidic lectin binding proteins in latex vessel plugging process and forseen a possible development of flow marker. Upon completion of marker developments, we plan to perform large trail by using biosynthetic and flow markers in selection of high-yield grafted-seedlings. The trail will be done under cooperation of the Songkla Rubber Research Center and by using the plantation site of the Bangkok Rubber Land and Engineering Company. Hopefully, we can come up with certified high-yield rubber seedlings for the farmers in the near future.

Project Activities/outputs:

Meetings attended:

1. PSTC Grantees Networking Meeting on "Tissue Culture" Networking/ Workshop Meeting " at UC-Davis, California, USA. 16-20 July 1990.
2. PSTC/CDR Networking Meeting on "Forestry Biotechnology Networking/Workshop Meeting" at E.A.R.T.H., Costa Rica. 1-7 April 1992.
3. The 4th Annual Meeting of Biotechnology Society of Thailand Bangkok, 29-31 October 1992.
4. The 18th. Conference on Science and Technology of Thailand Bankok, 28-31 October 1992.

Pulications:

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6. R. Wititsuwannakul, D. Wititsuwannkul, D. Sattayasevena and P. Pasitikul (1993) Bark Peroxidase from *Hevea brasiliensis*. (see enclosed manuscript II, to be submitted to the Phytochemistry Journal).

Project productivity:

The project did accomplished all the proposed goals.

Future Work:

In order to persue further study on protective role of bark peroxidase and latex phenols in stabilizing lutoids in latex, thus leading to an increase in latex stability and increasing its flow time upon tapping, a full proposal with I.D. # C-14-073, entitled "Markers in Early Selection of High-Yield Rubber Seeding" was recently submitted for USAID/CDR support consideration.

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