



A LECTIN FROM THE BARK OF THE RUBBER TREE (*HEVEA BRASILIENSIS*)

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Key Word Index—*Hevea brasiliensis*; Euphorbiaceae; bark; lectin.

Abstract—A protein isolated and purified from extracts of bark strips from mature rubber (*Hevea brasiliensis*) agglutinated erythrocytes from rabbits and all human blood types except AB. No agglutination was detected with erythrocytes from sheep, rates or mice. Proteolytic treatment of *Hevea* bark lectin (HBL) abolished haemagglutinin activity. The M_r determined by SDS-PAGE was 40 k and that estimated from gel filtration was 140 k. Fetuin, asialofetuin, bovine submaxillary mucin and asialosubmaxillary mucin inhibited HBL-induced agglutination of rabbit erythrocytes. The HBL showed maximum haemagglutination activity over the pH range 4.5–9.5 and heat stability up to 60°. © 1997 Elsevier Science Ltd

INTRODUCTION

Plant lectins are a heterogeneous class of proteins, or glycoproteins, that have in common, the ability to recognize specifically, carbohydrate ligands. Their widespread distributions throughout the plant kingdom suggests they are physiologically important. A number of roles have been proposed, for example in wall extension [1], in stimulation of plant cell proliferation and growth [2], carbohydrate transport [3], as a signal transducers in plant-microbe interactions [4], as storage proteins [5] and in plant defense [6]. Seeds, especially those of leguminous species, are common sources of lectins but they are also present in vegetative tissues such as stems, roots and latex of various species [3, 7–10]. Lectins have also been reported from the bark of several trees including three species of elder (*Sambucus nigra* L.) [8, 11], *S. canadensis* [12] and *S. sieboldiana* [13], golden chain (*Laburnum anagyroides*) [14], *Sophora japonica* [9] and the black locust (*Robinia pseudoacacia*) [15]. In black locust they are the most abundant bark proteins [16] and are present as complex mixtures [17]. In this paper we describe the isolation and characterization of a lectin from *Hevea* bark.

RESULTS AND DISCUSSION

A protein isolated from *Hevea* bark agglutinated erythrocytes from rabbits and has weak agglutinating

Table 1. Erythrocyte specificity of HBL from crude bark extract

Erythrocyte Source	Hemagglutination (titre*)
Rabbit	24
Human (Type A)	6
Human (Type B)	3
Human (Type AB)	0
Human (Type O)	4
Sheep	0
Rat	0
Mouse	0

* Titre is defined as the reciprocal of the lowest dilution which gave detectable agglutination of rabbit erythrocytes.

activity with human erythrocytes of all blood groups except AB, but no activity with erythrocytes from sheep, rats or mouse (Table 1). Proteolytic treatment of HBL with either trypsin, protease K or pronases destroyed all haemagglutinin activity (data not shown). For purification, the crude bark extract was first freed of contaminating polyphenolic compounds by an aqueous two-phase partitioning step. Further purification was achieved by successive chromatography on DEAE-cellulose (Fig. 1) and Biogel P-300 (Fig. 2) chromatography. The purified HBL had a specific titre against rabbit erythrocytes of 0.08 $\mu\text{g ml}^{-1}$ (Table 2). The M_r of purified HBL determined by gel filtration was ca 141 k. SDS-PAGE showed a single protein band with M_r ca 40 k (Fig. 3). Fetuin, asialofetuin, bovine submaxillary mucin and asialo

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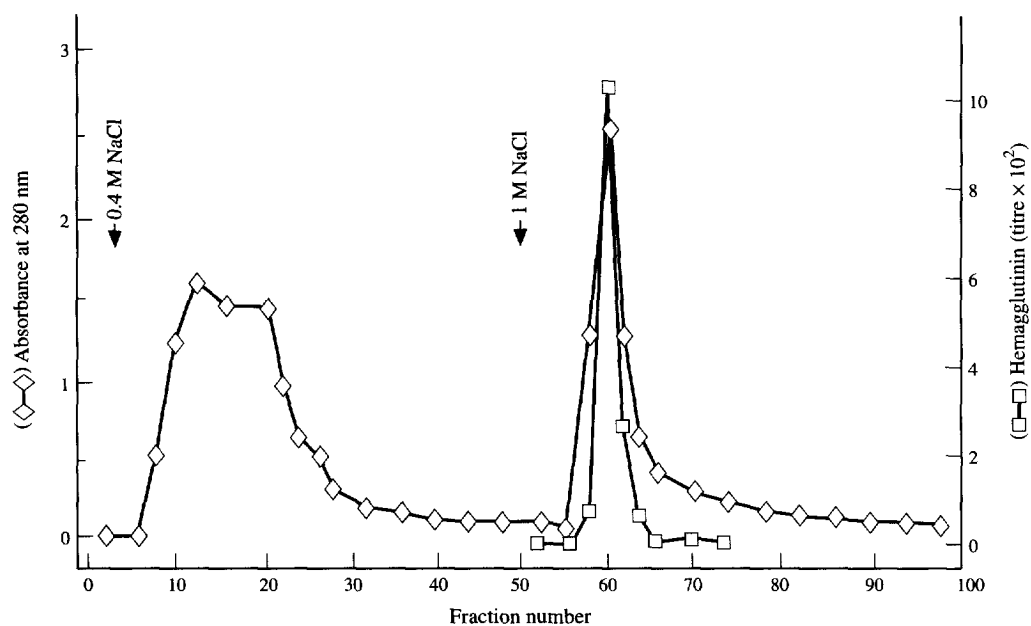


Fig. 1. DEAE-cellulose chromatograph elution profile of *Hevea* bark peroxidase and haemagglutinin activities.

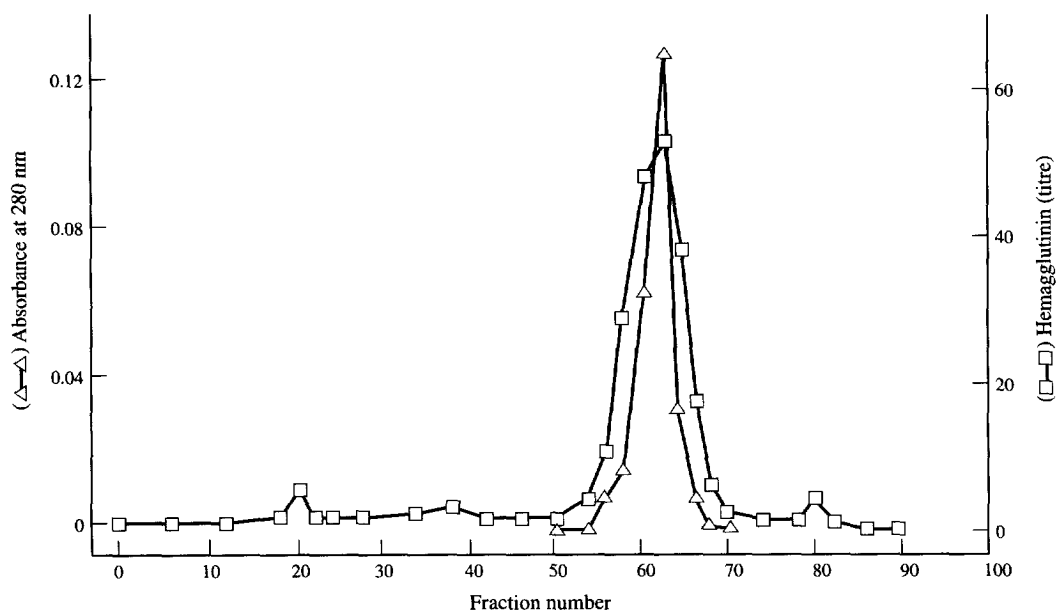


Fig. 2. Bio-gel P-300 chromatography of HBL from DEAE-cellulose column (see Fig. 1).

Table 2. Purification protocol of HBL

Fraction	Titre	Specific titre* ($\mu\text{g ml}^{-1}$)	Yield (%)
Bark extract	3.18×10^7	ND	100
Aqueous two-phase partitioning	6.75×10^4	1.17	0.21
DEAE-Cellulose Eluate (1.0 M NaCl)	2.91×10^4	0.27	0.09
Biogel P-300	1.26×10^4	0.08	0.04

ND, not determined due to contaminating phenolic interference in protein determination.

* Minimal concentration of HBL required for detectable haemagglutination.

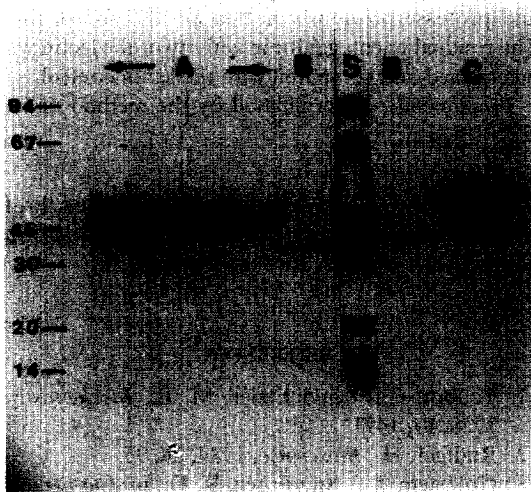


Fig. 3. SDS-PAGE of purified HBL (B), crude bark extract (A and C). Lane S, standard marker proteins. Gel stained with Coomassie Blue.

bovine submaxillary mucin inhibited HBL-induced rabbit erythrocyte agglutination, whereas a range of monosaccharides were without activity (Table 3). The precise carbohydrate-binding specificity of the HBL remains to be defined and *N*-acetylneuraminyl residues do not appear to be involved as ligands for the HBL. The haemagglutinin activity of HBL was heat stable up to 60°. A sharp decrease in its activity was observed when temperature was raised from 60 to 80°. The HBL-induced haemagglutination was not markedly affected by variation of the pH and its maximum activity was retained over the broad pH range 4.5–9.5.

Strip-wounding of rubber bark by tapping opens the latex vessels in the inner bark layer allowing the flow of fresh latex and is accompanied by bursting of the latex luteoids and release their contents (B-serum)

[18]. The extent of luteoid bursting, as indicated by the bursting index, has been reported to be inversely correlated with rubber yield [19, 20]. It has been suggested that luteoids play an important role in the formation of latex-vessel plugs which impede latex flow [18–20]. In order to assign specific *in vivo* roles to HBL, our current studies are aimed at testing the possibility that the HBL has specific binding with B-serum proteins from disrupted luteoids in initiating the *in vivo*-wounding response leading to the sealing of the latex vessel.

EXPERIMENTAL

Chemicals. *o*-Dianisidine, DEAE-cellulose, glycoproteins, sugars, proteases, Protease K, Protease XIV and trypsin were from Sigma. Pronase was from Calbiochem-Boehringer Corp., Bio-gel P-300 from Bio-rad and H₂O₂ from Merck. All other chemicals were of reagent grade.

Bark collection. Newly-excised bark strips were collected after tapping, the rubber strings remaining from previous tapping were removed and the string-free bark strips washed with H₂O and used immediately for lectin extraction.

Bark extract preparation. Rubber-free bark was homogenized in a Waring blender in 10 mM K-Pi buffer, pH 7 and the homogenate filtered through cheese cloth to remove the bark debris. The crude bark extract was sepd as a clear dark-brown supernatant fr. after centrifugation at 20 000 *g* for 1 hr. The extract was concd by ultrafiltration (Amicon, *M*, 10 000 cut-off). Polyphenolic compounds were removed by an aq. two-phase partitioning performed. PEG 8000 was added to the bark extract to a final conc of 10%. After complete dissolution of the PEG, K citrate was added to a final concn of 30%. On settling for 30 min, the mixt. sepd into two phases. The polyphenolic

Table 3. Carbohydrate binding specificity of HBL

Glycoprotein	Concentration required for 100% Inhibition of agglutination of rabbit erythrocytes in the presence of HBL (3 µg ml ⁻¹)
Fetuin	0.156
Asialofetuin	0.078
Bovine submaxillary mucin	0.313
Asialo bovine submaxillary mucin	1.230

The carbohydrate binding specificity of the HBL was determined by hapten inhibition assays using a series of monosaccharides and some glycoproteins. The following monosaccharides were not inhibitory; D-mannose (40 mM), D-mannosamine (40 mM), *N*-acetyl-β-D-mannosamine (30 mM), D-galactosamine (40 mM), *N*-acetyl-D-galactosamine (40 mM), *N*-acetyl-D-glucosamine (40 mM) and *N*-acetylneuraminic acid (10 mM).

compounds were found in the upper, PEG phase, and the lower, aq. layer contained the lectin protein.

DEAE-cellulose chromatography. The aq. layer from the two-phase sepn was dialysed by ultra-filtration (Amicon, *M*, 10 000 cut-off) and 2 ml of the filtrate loaded onto a DEAE-cellulose column (1.4 × 15 cm), previously equilibrated with buffer A (50 mM Tris-HCl, pH 7.4). Following extensive washing with buffer A, the column was eluted successively with 0.4 M and 1 M NaCl in buffer A.

Bio-gel P-300 chromatography. The lectin-containing frs. from the DEAE-cellulose chromatography were pooled, dialysed, and concd by ultrafiltration (Amicon, *M*, 10 000 cut-off) and loaded onto a Bio-gel P-300 column (1.4 × 50 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⁻¹ and 1 ml frs. were collected. The same column was calibrated with bovine serum albumin, aldolase and catalase as *M*, markers.

Hemagglutination assay. Hemagglutination assays were performed in U-well microtiter plates in a final vol. of 100 µl containing 50 µl of a 1.5% suspension of rabbit erythrocytes, previously washed × 3 in K-Pi buffered saline, and 50 µl of lectin solns (each serially diluted with 2-fold increments). Agglutination was recorded visually after 1 hr at room temp. as the reciprocal of the lowest dilution giving detectable agglutination. The carbohydrate-binding specificity of the lectin was determined by inhibition assay using the glycoproteins (fetuin, asialofetuin, bovine submaxillary mucin, asialo bovine submaxillary mucin) and a series of monosaccharides. The monosaccharides tested were: D-mannose, D-mannosamine, *N*-acetyl-β-D-mannosamine, *N*-acetyl-D-glucosamine, D-galactosamine, *N*-acetyl-D-galactosamine and *N*-acetylneuraminic acid.

Polyacrylamide gel electrophoresis. SDS-PAGE was carried out in 7.5–15% polyacrylamide gel according to ref. [21].

Effect of pH and temp. The effect of pH and temp. on haemagglutinin activity of HBL was determined by incubating the samples at various pHs for 1 hr or at defined temp. for 20 min as indicated. The mixts were adjusted back to pH 5.4 or 4° and assayed for haemagglutinin activity.

Effect of protease treatments. Partially purified HBL fraction (5 µg) obtained after aq. two-phase partitioning was incubated at 37° for 1 hr with each of the following enzymes; protease K 20 mg ml⁻¹ (12.2 U ml⁻¹), pronase XIV 50 mg ml⁻¹ (5.4 U ml⁻¹), pronase 50 mg ml⁻¹ (45 000 PUK), and trypsin 50 mg ml⁻¹ (9800 BAEE units ml⁻¹). The enzymes (20 µl of each) were added to the lectin in buffer (40 µl). After incubation, the mixts were immediately placed on ice, and assayed for residual haemagglutinin activity. Lectin and protease controls contained lectin or protease with H₂O instead of enzyme or lectin, respectively. The heat-inactivated enzyme control contained lectin

and protease previously heated at 100° for 10 min. The residual haemagglutinin activity of the lectin was determined and compared with the lectin control.

Protein conc. Determinated by the method of ref. [22].

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