Purification and Characterization of Two Major Lectins from Araucaria brasiliensis syn. Araucaria angustifolia Seeds (Pinhão)¹

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ABSTRACT

Two major lectins (lectin I and lectin II) were purified to homogeneity from the seeds of Araucaria brasiliensis (Gymnospermae). The purity of the lectins was confirmed by polyacrylamide gel electrophoresis, isoelectric focusing, and high-performance liquid chromatography. They are glycoproteins in nature containing 8.3 and 2.9%, respectively, of neutral sugar and have absorption coefficients of 3.8 and 4.7, respectively, at 280 nanometers. The molecular weights of both lectins obtained by gel filtration on Sepharcl S-400 were equal: 200,000. After dissociation by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, molecular weights were 20,000 and 34,000, respectively, for lectin I and lectin II, suggesting they are decameric and hexameric in nature. The amino acid composition of both lectins showed little difference, but both had high amounts of acidic amino acids and lacked methionine in their molecule. The carbohydrate binding specificity of lectins was directed towards mannose, glucose, and their oligomers. High inhibitory activity was also found with thyroglobulin. The erythrosagglutinating activity of the lectins was enhanced in the presence of high-molecular-weight substances both at 37 and 4°C. Divalent cations do not appear to be essential for activity. They maintained their agglutinating activity over a broad but different range of pH: 5.5 to 7.5 and 6.5 to 7.5, respectively. Both lectins agglutinated erythrocytes of human ABO blood types equally well.

 Lectins are a special group of sugar-binding proteins of nonimmune origin able to agglutinate cells and/or precipitate glycoconjugates (11). They have been identified in nature from microorganisms to animals (10) and display a wide variety of unique and interesting biological properties (18). Several hypotheses concerning their biological functions have been reported, but this remains an unsettled question. "Pinhão" is the seed of the tree "Pinheiro de Paraná"; it is consumed as food. This tree is a Gymnospermae that grows in the south of Brazil and in the northern region of Argentina. It belongs to the Coniferae group, which forms, nowadays, the biggest living group of Gymnospermae (21). No information was found in the literature on the presence and description of lectins in Gymnospermae. Here, we describe the isolation, purification, and some of the properties of the pinhão lectins.

MATERIALS AND METHODS

Pinhão, Araucaria brasiliensis, was purchased from a local supermarket. Normal typed human erythrocytes were obtained from the University Medical College Blood Bank (São Paulo, Brazil). HPLC grade methanol was from E. Merck (FRG). Bio-Gel P-150 was from Bio-Rad Laboratories (Richmond, CA). Pheny1-Sepharose CL-4B, DEAE-Sephadex, Pharmalite, (pH 3.0 to 10.0), and Sephacryl S-400 were all from Pharmacia Fine Chemicals (Uppsala, Sweden). The following chemicals were all obtained from Sigma Chemical Co. (St. Louis, MO): thyroglobulin, pepsin, arabinogalactan, polyvinylpyrrolidone, myosin from rabbit muscle (M₉, 205,000), β-galactosidase from Escherichia coli (M₉, 116,000), phosphorylase b from rabbit muscle (M₉, 97,400), BSA (M₉, 66,000), albumin from egg (M₉, 45,000), carbonic anhydrase from bovine erythrocyte (M₉, 29,000), β-lactoglobulin (M₉, 18,400), lysozyme (M₉, 14,300), D-xylene, D-mannitol, D-galactose, D-fructose, D-mannose, raffinose, lactose, D-glucosamine HCl, L-rhamnose, D-glucose, para-nitrophenyl-α-D-glucoside, para-nitrophenyl-β-D-glucoside, para-nitrophenyl-α-D-mannoside, para-nitrophenyl-N-acetyl-β-D-galactosaminide, para-nitrophenyl-N-acetyl-β-D-glucosaminide, para-nitrophenyl-α-D-galactoside, para-nitrophenylmaltsoside, para-nitrophenyl-β-D-fucoside and L-arabinose. All other chemicals were of analytical grade.

Purifications of Lectins

Step I. Dehusked pinhão (100 g) was soaked in 1000 mL of PBS (pH 7.2) (8 mM phosphate buffer containing 0.15 M NaCl) for 12 h at 4°C and was then homogenized. The homogenate was filtered through cheesecloth. The supernatant solution (crude extract) was collected after centrifugation for 30 min at 10,000g. The purification was continued from 30 mL of the crude extract, containing 82.2 mg of proteins (Table I). This extract was dialyzed overnight against Mcllvaine buffer (pH 4.8) in 0.2 M NaCl; protein precipitated during dialysis was centrifuged at 25,000g for 20 min. The precipitate was dissolved in 0.1 M phosphate buffer (pH 7.2) containing 0.20% sodium azide in 0.75 M NaCl at 25°C. This
solution was dialyzed and centrifuged as described above. The precipitate obtained on centrifugation was called GF-1. The supernatant obtained at this stage was pooled with the supernatant obtained in the previous step. The combined supernatants were dialyzed against deionized water with several changes for 24 h and centrifuged at 25,000g for 20 min. The precipitate was called GF-2. The supernatant at this stage still contained protein and was called albumin protein. The GF-1 and GF-2 preparations were dissolved in 0.2 M NaCl in 0.15 M phosphate buffer of pH 7.5.

Step II. Gel filtration of the albumin protein fraction was carried out on a Phenyl-Sepharose CL-4B (56 × 1.5 cm) column. The column was washed and equilibrated with PBS. The albumin protein fraction was concentrated and dialyzed against PBS and applied to the Bio-Gel P-150 column. Protein was eluted from the column with PBS at a flow rate of 12 mL/h, 3 mL/tube, and fractions showing agglutinating activity were pooled and concentrated.

Step III. The hydrophobic separation of the most active fraction on the gel filtration column was done on a Phenyl-Sepharose CL-4B (56 × 1.5 cm) column. The column was washed and equilibrated with 8 mM phosphate buffer (pH 7.2). The minor peak from gel filtration column was dialyzed against the same buffer, and, after being loaded into the hydrophobic column, protein fractions were eluted with a NaCl gradient (0 to 1.0 M) at a flow rate of 10 mL/h, 2 mL/tube. The proteins eluted at 0.16 M salt concentration possessed erythroagglutinating activity. The active fractions were pooled and concentrated.

Step IV. The concentrated fraction was then applied to a DEAE-Sephadex column. Before application of the protein, the column was washed and equilibrated with 8 mM phosphate buffer (pH 7.2). The protein was dialyzed against the same buffer added to the column and was washed with 20 mL of the same buffer. Afterward, a linear gradient of NaCl (0 to 0.5 M) was started at a flow rate of 20 mL/h, 1 mL/tube. The peaks showing hemagglutinating activity were pooled separately and concentrated.

Step V. HPLC on a LKB (Bromma, Sweden) 2150 HPLC pump system was used for further analysis of the lectins. The individual peaks obtained from the DEAE-Sephadex column were dialyzed against PBS, and a portion containing 50 µg of protein was applied to the HPLC column (Ultracap TSK G-3000 SW) previously equilibrated with PBS. The column effluent, at a flow rate of 0.5 mL/min, was monitored at 280 nm.

Electrophoresis

PAGE of the purified lectins was performed by the Davis method (6). The molecular mass of the lectins treated with SDS in the presence of 2-mercaptoethanol was determined (14) by using standard Mr markers.

Isoelectric Focusing

Isoelectric focusing was carried out in a rod gel (12 × 0.6 cm) containing 4% ampholine (pH 3.0 to 10.0) and 10% polyacrylamide (12). After a run for 6 h at 300 V, the gel was cut into slices, the slices were immersed in 1 mL of water, and the pH of the extract was determined. Lectin activity in each slice was then measured.

Protein Estimation

The protein was determined by the method of Lowry et al. (19). Eluates from the columns were also monitored spectrophotometrically at 280 nm.

Hemagglutination Assay

Hemagglutination was assayed in V-microtiter plates (4) by serially diluting a 50-µL sample into 50 µL of PBS. Added to each well was 50 µL of 2% rabbit erythrocyte suspension in the same buffer, and hemagglutination was determined after a 1-h incubation at 37°C as the reciprocal of the greatest dilution that gave visible aggregation. Effects of saccharides, glycoprotein, arabinogalactan, and polyvinylpyrolidone on hemagglutination were examined by preincubation of the lectin with the test substance at 37°C for 30 min, followed by incubation at 37°C for 1 h after addition of erythrocyte suspension. Agglutinating activity of these high-molecular-weight polymers in the absence of lectins was determined by the addition of 50 µL of PBS, 50 µL of polymer solution (1 mg/mL), and 50 µL of 2% rabbit erythrocyte suspension to each well and by the incubation of the plates for 1 h at 37°C.

Molecular Mass Determination

The molecular weights of lectin I and lectin II were determined (2) by gel filtration on a Sephacryl S-400 column (70 × 1 cm; 1 mL/tube; flow rate, 10 mL/h) calibrated with myosin (Mr, 205,000), β-galactosidase (Mr, 116,000), phosphorolysis b (Mr, 97,400), BSA (Mr, 66,000), ovalbumin (Mr, 45,000), and carbonic anhydrase (Mr, 29,000).

Divalent Cation Requirement

The purified lectins were dialyzed against PBS (pH 7.2) containing 10 mM EDTA. Excess EDTA was removed by extensive dialysis against the same buffer. Hemagglutinating activity was assayed on the dialysis residue alone and in the presence of CaCl2, MnCl2, and MgCl2 at concentrations varying from 10 to 40 mM.

Carbohydrate Analysis of the Agglutinating Proteins

The neutral sugar content of the purified lectins was estimated by the method of Dubois et al. (7) with D-glucose as a standard.

Effect of pH and Temperature

The following were used for pH studies: acetate buffers, pH 4.0 to 6.5; phosphate buffers, pH 6.5, 7.2, and pH 7.5 to 8.0; borate buffers, pH 8.5 to 9.5; glycine buffers, pH 9.5 to 10.5. All buffers contained 0.15 M NaCl, and pH intervals of 0.5 units each.

The effect of temperature on hemagglutination activity of lectins was determined by incubating the lectin samples for

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2 Abbreviation: GF, globulin fraction.
30 min at different temperatures from 25 to 80°C at 10°C increments.

**Amino Acid Analysis**

The amino acid analysis of purified lectin I and lectin II was performed by the method of Spackman et al. (22) on a Beckman 121 MB automatic amino acid analyzer (Beckman Instrument Co., Fullerton, CA) equipped with a Hewlett Packard Integrator (Model HP3396A) after the hydrolysis of the samples in 6 M HCl for 24 h at 110°C. Cysteine and methionine were analyzed after performic acid oxidation and HCl hydrolysis. Tryptophan was calculated by the method of Edelhoch (8).

**RESULTS AND DISCUSSION**

The purification steps are depicted in Table I. The seed coat had no agglutinating activity; maximum lectin activity was recovered from the albumin fraction. This fraction by chromatography on Bio-Gel P-150 yielded one minor and one major peak (Fig. 1). The minor peak showed maximum lectin activity and was purified further through a Phenyl Sepharose CL-4B column. This gave only one major peak with high lectin activity, but it was found heterogeneous on electrophoresis. For further purification, this fraction was applied to DEAE-Sephadex column. With a 0 to 0.5 M NaCl gradient, we obtained 10 peaks, 6 of them having hemagglutinating activity. Among them, the last two peaks presented 90% of total activity. They were called lectin I and lectin II and were purified by TSK G-3000 SW HPLC (Fig. 2). Lectin I and lectin II eluted as single symmetrical peaks and were found to be homogeneous with 117.9-fold and 76.7-fold purification, respectively, and with high specific activities (Table I). The minimum amount of purified lectin that gives positive agglutination was 0.0028 μg/mL for lectin I and 0.0043 μg/mL for lectin II. In our laboratory under similar conditions, for *Phaseolus vulgaris*, soybean, etc., we obtain values from 1 to 7 μg/mL approximately.

Generally, seeds contain one lectin, even though there are some reports on the presence of more than one (3, 13, 15, 17). It is possible that plants contain families of lectin genes that may have altered patterns of expression in a tissue or in different tissues of the same plant. Common lectin gene products may also differ during posttranslational modification (9).

### Table I. Purification Scheme of Lectins from A. brasiliensis Seeds

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Total Protein</th>
<th>Specific Activity</th>
<th>Total Activity</th>
<th>Purification</th>
<th>Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mg</td>
<td>titer/mg of protein × 10^-3</td>
<td>titer × 10^-3</td>
<td>-fold</td>
<td>%</td>
</tr>
<tr>
<td>Crude extract*</td>
<td>82.2</td>
<td>29.9</td>
<td>2457.8</td>
<td>1</td>
<td>100</td>
</tr>
<tr>
<td>GF-1</td>
<td>20.0</td>
<td>0.6</td>
<td>12.0</td>
<td>0.0</td>
<td>0.5</td>
</tr>
<tr>
<td>GF-2</td>
<td>14.5</td>
<td>2.6</td>
<td>37.7</td>
<td>0.1</td>
<td>1.5</td>
</tr>
<tr>
<td>Albumin proteins</td>
<td>23.8</td>
<td>97.2</td>
<td>2294.0</td>
<td>3.3</td>
<td>93.3</td>
</tr>
<tr>
<td>Bio-Gel P-150</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Peak (minor)</td>
<td>3.5</td>
<td>420.1</td>
<td>1470.4</td>
<td>14.1</td>
<td>59.8</td>
</tr>
<tr>
<td>Peak (major)</td>
<td>17.6</td>
<td>12.8</td>
<td>225.3</td>
<td>0.4</td>
<td>9.2</td>
</tr>
<tr>
<td>Phenyl-Sepharose</td>
<td>1.4</td>
<td>963.8</td>
<td>1349.3</td>
<td>32.2</td>
<td>54.9</td>
</tr>
<tr>
<td>DEAE-Sephadex</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Peak I</td>
<td>0.1</td>
<td>25.8</td>
<td>2.6</td>
<td>0.9</td>
<td>0.1</td>
</tr>
<tr>
<td>Peak II</td>
<td>0.1</td>
<td>6.8</td>
<td>0.7</td>
<td>0.2</td>
<td>0.0</td>
</tr>
<tr>
<td>Peak III</td>
<td>0.3</td>
<td>157.5</td>
<td>47.3</td>
<td>5.3</td>
<td>1.9</td>
</tr>
<tr>
<td>Peak IV</td>
<td>0.2</td>
<td>63.2</td>
<td>12.6</td>
<td>2.1</td>
<td>0.5</td>
</tr>
<tr>
<td>Peak V</td>
<td>0.2</td>
<td>2978.9</td>
<td>595.8</td>
<td>99.6</td>
<td>24.2</td>
</tr>
<tr>
<td>Peak VI</td>
<td>0.3</td>
<td>2048.0</td>
<td>614.4</td>
<td>68.5</td>
<td>25.0</td>
</tr>
<tr>
<td>HPLC</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Peak V (lectin I)</td>
<td>0.2</td>
<td>3524.6</td>
<td>704.9</td>
<td>117.9</td>
<td>28.7</td>
</tr>
<tr>
<td>Peak VI (lectin II)</td>
<td>0.2</td>
<td>2292.7</td>
<td>458.5</td>
<td>76.7</td>
<td>18.7</td>
</tr>
</tbody>
</table>

* 30 mL.
CHARACTERIZATION OF LECTINS FROM ARAUCARIA BRASILIENSIS SEEDS (PÍNHÃO)

Figure 2. HPLC assay of protein from peak V (a) and peak VI (b) obtained from DEAE-Sephadex column. The sample protein (50 μg in 100 μL) was applied to the column. The column effluent was monitored at A280 = 0.1 absorbance unit full scale. Inset: PAGE from lectin I (a) and lectin II (b), 30 μg of protein in each gel tube, 5 mA/gel (7.5%).

The homogeneity of the purified lectins, lectin I and lectin II, were confirmed by several criteria. On PAGE at pH 8.3 (Fig. 2) and SDS-PAGE (Fig. 3), they gave a single protein band. The $M_r$ values of lectins I and II by SDS-PAGE were calculated to be 20,000 ± 1,000 and 34,000 ± 1,000, respectively (Table II). The homogeneity of the purified lectins was also confirmed by isoelectric focusing. Both showed a single band at pH values of about 7.3 and 7.5, respectively (Table II).

Gel filtration on Sephacryl S-400 column of lectin I resulted in one major and four minor peaks; the $M_r$ of the major peak was 200,000 ± 1,000 and the minor peaks were 79,000 ± 1,000; 59,500 ± 1,000; 39,000 ± 1,000 and 20,000 ± 1,000, respectively. This suggests that lectin I exists as a decamer in the native stage with aggregation of one single monomer. However, lectin II, on Sephacryl S-400 gel filtration, showed two peaks with $M_r$ values of 200,000 ± 1,000 and 68,000 ± 1,000, respectively, which indicates that it probably exists as a hexamer formed by the association of three dimers (Table II). Minor polypeptide fragments may result from different association of subunits.

Table II. Characteristics of Lectin I and Lectin II

<table>
<thead>
<tr>
<th>Properties</th>
<th>Lectin I (M_r)</th>
<th>Lectin II (M_r)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Native $M_r$</td>
<td>200,000 ± 1,000</td>
<td>200,000 ± 1,000</td>
</tr>
<tr>
<td>Subunit $M_r$</td>
<td>20,000 ± 1,000</td>
<td>34,000 ± 1,000</td>
</tr>
<tr>
<td>Possible structure</td>
<td>Decamer</td>
<td>Hexamer</td>
</tr>
<tr>
<td>Isoelectric point</td>
<td>~7.3</td>
<td>~7.5</td>
</tr>
<tr>
<td>Neutral sugar (%)</td>
<td>6.3</td>
<td>2.9</td>
</tr>
<tr>
<td>$A_{280}^\text{nm}$ at 280 nm</td>
<td>3.8</td>
<td>4.7</td>
</tr>
</tbody>
</table>

Figure 3. SDS-PAGE in presence of 2-mercaptoethanol of lectin I (a) and lectin II (b) at pH 8.3. Agglutinating protein (50 μg) was applied in each gel tube. A current of 3 mA/gel (10%) was applied for 8 h at 10°C.
Measurements of the molecular weights of lectins by gel filtration may be subject to some uncertainty owing to interactions in the lectin matrix, but, in most cases, good estimates were possible by this technique.

Lectins I and II are glycoproteins in nature, containing 6.3 and 2.9% neutral sugars (Table II).

The absorption coefficients A\textsubscript{1% cm} of lectins I and II were 3.8 and 4.7, respectively (Table II).

In the presence of high-molecular-weight substances, both lectins showed different behavior at 37°C (Table III). Specific activity of lectin I increased in the presence of arabinogalactan, but it was not affected by PVP, whereas specific activity of lectin II was increased by the presence of both substances. In the absence of lectin, these substances did not present agglutinating activity.

At low temperature, interesting results were obtained. At 4°C, the activity of neither lectin changed but, at this tempera-
Both lectin I and lectin II agglutinated erythrocytes of human ABO blood types equally well.

*A. brasiliensis* lectins are interesting substances because of their high specific activities and their structural features.

In this article, we have described some properties of these lectins, which have two main characteristics that have not been found in plant lectins before: extremely high agglutinating activity and their presence in Gymnospermae.

The high agglutinating activity observed in lectins I and II may be explained by the numerous subunits presented by them (decameric and hexameric, respectively), creating a high valence number and, thus, a high possibility of binding.

Hundreds of lectins have been recognized in plants, some of them already have been purified and some are being commercialized. However, the presence of lectins in Gymnospermae had not been described before. *A. brasiliensis* lectins could be used for taxonomic and phylogenetic studies and, what is more, for evolutionary correlation studies (16).

The amino acid compositions of lectins are presented in Table V. Analysis revealed high amounts of acidic amino acids, glycine, cysteine, and lysine for lectin I. In lectin II, acidic amino acids, threonine, glycine, and valine were higher in content when compared with other amino acids. The content of aromatic amino acids was higher in lectin II than in lectin I. Methionine was absent in both lectins.

Purified lectin I and lectin II exhibited maximal specific activities between pH 5.5 to 7.5 and pH 6.5 to 7.5, respectively (Fig. 4). No significant difference was observed with different buffers at overlapping pH values in both the cases.

The thermal stability of the lectins is shown in Figure 5, where the change of specific activities is depicted. Lectin II was more temperature labile than lectin I. With increasing temperature, both lectins lost their activities, which essentially cease at 80°C.

**Figure 4.** pH dependence activity profile of *A. brasiliensis* lectin I (A) and lectin II (B). ■, Acetate buffer; ▲, phosphate buffer; ●, borate buffer; O, glycine buffer. For experimental details, see the text.

**Figure 5.** Temperature dependence activity profile of *A. brasiliensis* lectin I (A) and lectin II (B). For experimental details, see the text.
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LITERATURE CITED