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Structural and biological features of a hydrogel from seed coats of *Chorisia speciosa*

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Abstract

Seed coats from *Chorisia speciosa* form a hydrogel on contact with water. When the hydrogel was solubilized and the solution centrifuged, subsequent ethanol precipitation gave a polysaccharide (F-I) composed of rhamnose, galactose and uronic acid in a molar ratio of 25:44:31. Analysis of F-I by HPSEC-MALLS showed a homogenous polymer with high molecular weight. It consisted of a main chain of (1→4)-linked β-galactopyranosyl units as indicated by NMR spectral and methylation data analysis, with rhamnose, galactose and glucuronic acid as non-reducing end units. This fraction interfered with adhesion of *Colletotrichum graminicola*, a causal agent of anthracnose, to polystyrene slides and to leaves of corn, thus delaying infection in the latter.

Keywords: *Chorisia speciosa*; Bombacaceae; Seed polysaccharides; Acidic galactan; Hydrogel

1. Introduction

Chorisia speciosa St. Hil. is a large tree of the family Bombacaceae, native to tropical and subtropical South America. Several aspects of this tree have been studied. For example, Lufrano and Caffini (1981) compared the composition of the mucilage formed by the leaves of four distinct species of *Chorisia* and suggested a chemotaxonomical relationship. The gum exudate, which appears when the trunk suffers injury, contains a complex polysaccharide with a backbone composed of glucuronosyl and mannosyl units (Di Fabio et al., 1982). The fruit of *C. speciosa* also produce an exudate when subjected to mechanical injury. This is an acidic arabinogalactan whose main chain is composed of (1→3) and (1→6) linked β-galactopyranosyl units (Beleski-Carneiro et al., 1999). Previous work showed that the silk floss from the seeds yielded, on alkaline extraction, a (1→4) linked β-D-xylan, substituted at O-2 by 5% of uronic acid (Beleski-Carneiro et al., 1996). The seeds contain 22% oil, which is predominantly unsaturated (Petronici et al., 1974). Defatted seeds furnished, on aqueous extraction, a viscous fraction with high content of protein (~40%) (Beleski-Carneiro et al., 1996). When the seeds were separated into seed coats and kernel, the seed coats swelled, forming a thick acidic polysaccharide hydrogel.

Biological activities of polysaccharides have also been reported. A hydrogel obtained from seed coats of *Magonia pubescens* (Tingui) was considered to be a germination promoter (Gorin et al., 1996). Experiments performed with *Coffea arabica* plants showed that solutions of (1→4)-linked β-polysaccharides induced systemic protection against its pathogen *Hemilea vastatrix* (Guzzo et al., 1993). Algal polysaccharides induced signaling and defense gene expression in tobacco plants (Mercier et al., 2001).

The establishment of a fungal pathogen on the surface of its host is essential to the success of the infection process. Conidia of *Colletotrichum graminicola*, the causal agent of corn anthracnose, adhere to hydrophobic surfaces immediatly after contact with the tissue and hours before the onset of germination. Conidia rarely adhere to hydrophilic surfaces (Mercure et al., 1994b).

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The structural features of the hydrogel obtained from the seed coats of *C. speciosa* and its biological effects in the interaction between corn (*Zea mays*) and its pathogen *C. graminicola* are now reported.

2. Results and discussion

2.1. Structural studies

Isolated seed coats were kept in water until swelling took place. The resulting hydrogel was solubilized in water and ethanol precipitation gave rise to fraction F-I (11% yield relative to isolated seed coats). The hydrogel contained protein (6%) and carbohydrates, with its monosaccharide composition being rhamnose, galactose and uronic acid in a molar ratio of 25:44:31. Minerals detected by atomic absorption were principally K (35.5 μg mg^{-1}) with Ca, Mg and Na in lower quantities (less then 5 μg mg^{-1}). Repeated solubilization in water, centrifugation and precipitation of F-I, removed neither protein nor minerals. The hydrogel was analysed by size exclusion chromatography (SEC), which indicated a molecular weight (M_w) and radius of gyration (R_g) of 2.77×10^6 and 146.4 nm, respectively. The mass distribution showed a homogenous polymer (Fig. 1) and the relationship between $R_g\times M$ suggested the absence of aggregates, or dissociated fragments under the experimental conditions used.

To determine the general structure of the mucilage, the polysaccharide was methylated by the method of Ciucanu and Kerek (1984) (Table 1). Methylation analysis and GC–MS spectrometric examination of the resulting partially *O*-methylated alditol acetates showed 2,3,6-Me_3-Gal (39%), suggesting that the main chain is composed of (1→4)-linked galactopyranosyl units. Rhamnose and galactose are present as terminal non-reducing units, as indicated by the derivatives 2,3,4-Me_3-Rha (12%) and 2,3,4,6-Me_4-Gal (3%), respectively. Other Rha derivatives are 2-linked (3,4-Me_2-Rha,

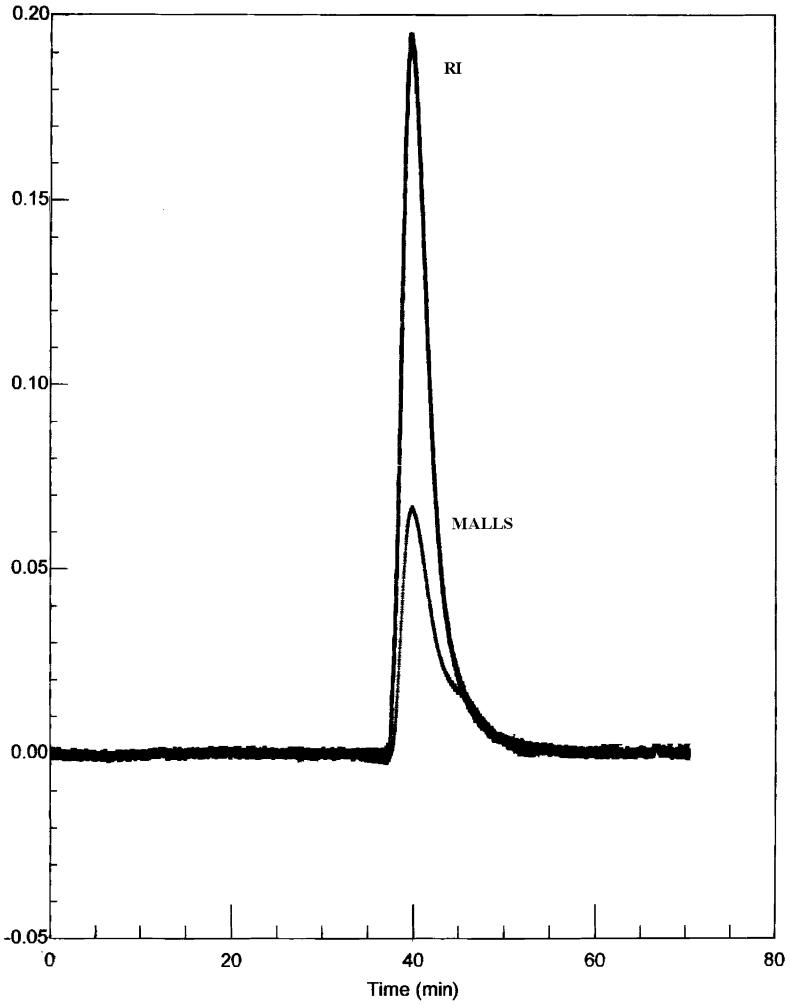

Fig. 1. High performance size exclusion chromatography (HPSEC) of the fraction F-I, with light scattering (MALLS) and refractive index (RI) detection.

5%) and 2,4-linked (3-Me-Rha, 23%). The latter could be from a branched side-chain. The polysaccharide was carboxyl-reduced by a carbodiimide variation (Redgwell et al., 1988) of the method of Taylor and Conrad (1972) converting the uronic acid residues to the corresponding 6,6-dideuterio-glycosyl residues. The product was methylated, converted into partially methylated alditol acetates, and examined by GC–MS. A C-6,6-dideuterio-labeled 2,3,4,6-Me_4-Glc derivative was detected (21%, m/z 131, 147 and 207), indicating the occurrence of glucuronic acid as terminal non-reduced units in the original polysaccharide. None of the deuterated galactose derivatives were detected. Considering the amount of uronic acid in the original fraction (~30%), the carboxyl-reduction was efficient (Redgwell and Selvendran, 1986).

The polysaccharide was analyzed by ^{13}C NMR spectroscopy (Table 2) and assignments are based on literature data (Gorin and Mazurek, 1975). The signals at extreme fields can be assigned to C-6 of two types of rhamnopyranosyl units (16.98 and 17.14 ppm) and to C-6 of α-glucuronic acid (175.31 ppm). However, *O*-methyl signals were not found in the ^{13}C and ^{1}H spectra, indicating the absence of 4-*O*-Me-Glc*p* residues. In the range of the anomeric region resonances (90–110 ppm), the signals at 105 and 104.3 ppm were assigned to C-1 of β-galactopyranosyl, at 100.83 to C-1 of α-glucuronic acid, and at 98.38 and 98.78 ppm to C-1 of α-rhamnopyranosyl residues. Signals at 77.93 and 76.84 ppm, are from C-4 substituted β-galactopyranosyl residues, typical of a (1→4) linked β-Gal*p* polysaccharide. The signals at 60.40 and 61.52 ppm indicate non substituted C-6 galactosyl units. Other peaks were assigned (Table 2), as 73.4 to C-4 of α-glucuronic acid, 69.68 and 70.54 ppm to C-2 of β-galactopyranosyl, both non substituted units. The multiplicity of peaks observed for C-4 atoms of galactose and rhamnose (77.93–76.84 ppm) suggested different environments available to the C-4 atoms. These signals confirmed methylation data.

This structure contrasts with those of most galactans that have been previously reported. For example, in primary cell walls extracted from seeds of white lupus (*Lupinus albus*) (Hirst et al., 1947) and other sources (McNeil et al., 1984), some of the galactans appear to have 6-O-substituents galactosyl in addition to 4-O-substituent galactosyl residues. These residues are present as side chains of pectic polysaccharides (RG-I and RG-II), generally as neutral segments with low molecular weight (Voragen et al., 1995).

Galactose is a common constituent of other cell wall polymers, such as arabinogalactans. In the type I arabinogalactans, the main chain is (1→4) linked and in the type II, it is (1→3) and (1→6) linked (Stephen, 1983). Although some were characterized over 30 years ago, the number of D-galactans that have been isolated and purified is not great, often due to the problem of separation from other polysaccharides. Significantly, larger amounts of neutral galactans can be extracted from cell walls of potato and onion with hot water; these galactans may have been a breakdown product of a more complex pectic polymer. However, in view of the mild conditions of extraction, it is believed that the small amounts of galactans from onion were native to the walls and not artefacts (Redgwell and Selvendran, 1986). Galactans with low contents of uronic acid have been described. Those from red spruce are slightly branched and appear to contain both glucuronic and galacturonic acids. Some glucuronic acid residues are also present in a related polysaccharide extracted from delignified tamarack (*Larix laricina*) (Stephen, 1983).

(1→4) linked D-galactans are also present in many seed cell walls as storage tissues and are mobilized before germination (Reid and Edwards, 1995). After germination, the galactan, and most of the arabinose, are

Table 1
Structure and percentage values of partially *O*-methylated alditol acetates formed from per-*O*-methylated polysaccharides from native (F-I) and carboxyl reduced hydrogel of seed coats of *Chorisia speciosa*

O-methylated alditol acetates	R_t (s) DB-210	% of *O*-methylated alditol acetate		
		F-I	Reduced F-I	Linkage
2,3,4-Me_3-Rha	493	12	8	Rha*p*-(1→
3,4-Me_3-Rha	523	5	2	2→)-Rha*p*-(1→
2,3,4,6-Me_4-Glc[a]	551	–	21	Glc*p*-(1→
2,3,4,6-Me_4-Gal	567	3	3	Gal*p*-(1→
3-Me-Rha	646	23	15	2,4→)-Rha*p*-(1→
2,3,6-Me_3-Gal	730	39	36	4→)-Gal*p*-(1→
3,4-Me_2-Gal	840	5	3	2,6→)-Gal*p*-(1→
2,3-Me_2-Gal	930	13	12	4,6→)-Gal*p*-(1→

Standard tested on DB-210 column was 2,3,4,6-Me_4-Glc (552 s).
[a] 6,6-dideuterio-labeled peak.

Table 2
^{13}C NMR spectroscopy data[a] of galactose, rhamnose and glucuronic acid in F-I fraction from seed coats of *Chorisia speciosa*

Monosaccharide constituents	Chemical shifts (ppm)					
	C-1	C-2	C-3	C-4	C-5	C-6
β-Gal	105.0 104.3	70.54 69.68	71.82 71.70	69.40 (76.84) (77.93)	[b]	61.52 60.40 (68.10)
α-Glc A	100.83	74.40	75.40	73.40	76.30	175.31
α-Rha	98.38 98.78	70.90 70.50 (76.70)	71.80 71.90	72.58 72.58 (77.80)	69.70	17.14 16.98

Figures in parentheses are assigned to C-linked.
[a] Values relative to the signal of DDS.
[b] Signal not well resolved.

mobilized from the wall, leaving a residue rich in galacturonic acid, rhamnose, and glucose (McNeil et al., 1984).

2.2. Biological effects of the hydrogel from the seed coats of C. speciosa

C. graminicola (Ces.) G. W. Wills is the causal agent of the anthracnose of cereals and grasses and is a disease of worldwide importance, especially because of its action on *Z. mays* (corn) plantations. The disease affects all parts of the plant and can be found at any time during the growing season, but is observed most frequently in the form of anthracnose leaf blight or stalk rot (Bergstrom and Nicholson, 1999). An important event in plant infection by the fungus is its adherence to the host surface. *C. graminicola* releases an extracellular matrix, which is supposed to perform this function (Mercure et al., 1995; Sugui et al., 1998). *Collectotrichum graminicola* requires a hydrophobic surface for the initiation of this infection process (Nicholson and Kunoh, 1995). An acidic hydrophilic compound could change the nature of the host surface. Considering this facts, the hydrogel isolated from seed coats of *C. speciosa* (F-I fraction) and the acidic arabinogalactan obtained from the fruit exudate (E-I) were tested to evaluate any interference in the interaction between *Z. mays* and its pathogen, *C. graminicola*.

2.2.1. In vitro experiments

It was observed (Mercure et al., 1994b) that when a conidial suspension from *C. graminicola* was applied on to an artificial hydrophobic surface, the conidia that adhered to it germinated, forming appressoria after 12 h incubation, which could not be removed by rinsing with distilled water. The extracellular matrix released from *C. graminicola* when incubated on an artificial hydrophobic substrate remained attached to the surface, suggesting that it has a role in adhesion (Sugui et al., 1998).

In the control experiment, a conidial suspension was applied on polystyrene slides, incubated, washed with distilled water and observed by microscopy. There were about 43 conidia in 0.2 mm^2, and 90% germinated to form melanized apressoria. When a conidial suspension was mixed with fraction E-I (exudate from fruit), the results were similar to those of the control. However, when a conidial suspension was first mixed with fraction F-I (hydrogel from seed coats), (0.62 mg/ml) and then applied to the surface, very few conidia adhered and germinated (4 conidia, in 0.2 mm^2), showing a significant interference (variance analysis 4% and Student Test 5%). This polysaccharide was used to perform in vivo experiments.

2.2.2. In vivo experiments

A second set of experiments was carried out using *Z. mays* leaves as a natural surface. Ungerminated conidia of *C. graminicola* adhere to corn leaves within minutes of contact (Mercure et al., 1994a). Leaves of 7-day-old plants inoculated with a conidial suspension showed signs of infection (tissue necrosis) 3 days after inoculation (Fig. 2). In contrast, leaves inoculated with a conidial suspension mixed with F-I polysaccharide (0.62 mg/ml) did not show signs of infection until after 7 days (Fig. 3). In another experiment in which the polysaccharide was first applied to the leaf surface, allowed to dry and the conidial suspension then applied at the same spot, infection was equally delayed.

The results suggest that the homogenous high molecular weight polysaccharide, characterized as a galactan containing glucuronic acid and rhamnose, delays infection by interfering fungal adhesion. By contrast, the other polysaccharide isolated from *C. speciosa* (E-I, from fruit exudate), in spite of its high molecular weight

Fig. 2. Leaf from *Zea mays* 3 days after inoculation with conidial suspension of *Colletotrichum graminicola*.

Fig. 3. Leaf from *Zea mays* 3 days after inoculation with conidial suspension mixed with F-I polysaccharide.

and acidic properties common to F-I, did not confer protection in this biological model and under experimental conditions used, suggesting that this biological property is related to the fine structure of the polysaccharide.

3. Experimental

3.1. Plant material

The seeds of *C. speciosa* were collected from large trees in parks of Curitiba-PR. The species was identified and sample voucher n° 32960 is deposited in the Herbarium (UPBC), Departamento de Botânica, Universidade Federal do Paraná, Curitiba, State of Paraná, Brazil.

3.2. Polysaccharide isolation

The seeds were separated into seed coats and kernel. The kernel was milled, defatted by Soxhlet extraction (72 h) with toluene: EtoH (2:1, v/v), submitted to enzyme inactivation with MeOH:H_2O (4:1, v/v) for 20 min, and immediately cooled and centrifuged. The insoluble residue was centrifuged for 10 min at high speed and extracted with H_2O water at 25 °C for 6 h. The residue was then extracted with KOH (2 M, 5 °C) and $NaBH_4$ under atmosphere of N_2 and dialysed. The extracts were treated with excess EtOH, the resulting precipitates dissolved in H_2O water and lyophilized. The seed coats were submitted to enzyme inactivation (as above) and to sequential extraction with H_2O yielding fraction F-I, and aqueous alkali to give fraction F-II.

3.3. General

Polysaccharides were hydrolyzed with 1 M TFA (5 h, 100 °C), the hydrolysates evaporated, the residues reduced with $NaBH_4$, and the products acetylated with pyridine–acetic anhydride (1:1 v/v, 16 h, at 25 °C). The resulting alditol acetates were analyzed by GLC (gas–liquid chromatography) using a model 5890 S II HP Gas Chromatograph at 220 °C (FID and injector temperature, 250 °C) using a DB-210 capillary column (0.25 mm i.d.×30 m), film thickness 0.25 μm, the carrier gas being nitrogen. ^{13}C NMR spectroscopy was performed with a Bruker DRX-400 spectrometer (100 MHz) in the Fourier transform mode, with complete proton decoupling at 80 °C, using D_2O as solvent in a 0.5 cm i.d tube. The spectral width was 200 ppm. Chemical shifts are expressed in ppm relative to the resonance of DDS (sodium 4,4-dimethyl-4-silopentane-1-sulphonate), used as internal standard ($\delta = 0$). Uronic acid was estimated by the *m*-hydroxybiphenyl (Blumenkrantz and Asboe-Hansen, 1973), total carbohydrate by the phenol-sulphuric acid (Dubois et al., 1956), and protein by the Hartree (1972) and Peterson (1977) methods. Determination of Ca, Mg, K, Na, Fe, Cu, Mn and Zn was carried by atomic absorption spectroscopy in a Perkin-Elmer 4100 spectrometer.

3.4. Determination of M_w of F-I fraction

A Waters size exclusion chromatography (SEC) apparatus coupled to a differential refractometer (RI), and a Wyatt Technology Dawn-F Multi-Angle Laser Light Scattering (MALLS) detector was used for characterization of fraction F-I (2 g l^{-1}). Four Waters Ultrahydrogel 2000/500/250/120 were connected in series and coupled with a multidetection equipment. A 0.1 M $NaNO_3$ solution, containing NaN_3 (0.5 g l^{-1}) was used as eluent. The specific refractive index increment was determined by using a Waters 2410 refractive index detector. The value of dn/dc (differential refractive index increment of the solvent–solute solution with respect to a change in solute concentration) was 0.173 at 633 nm.

3.5. Carboxyl reduction of F-I fraction

Carboxyl groups from uronic acid residues of fraction E-I (20 mg) were reduced to corresponding 6,6-dideuterio-glycosyl residues by a variation (Redgwell et al., 1988) of the method of Taylor and Conrad (1972). Polysaccharide (20 mg) was added in a beaker containing urea (4.8 g) and kept overnight under vacuum over P_2O_5 at 40 °C. D_2O (8 ml) was added and the contents were stirred until the polysaccharide dissolved. The pH was adjusted to 4.75 with 0.1 M DCl and maintained at this pH while 1-cyclohexyl-3-(2-morpholinoethyl) carbodiimide metho-*p*-toluensulfonate (CMC; 400 mg) was added for 30 min. The solution was stirred and (500 mg) $NaBD_4$ was added for 30 min. The pH was adjusted to 7.0–7.5 with 4 M DCl. After 2 h, the solution was acidified with HCl (pH 4.0), dialyzed and freeze-dried. The reduction was repeated.

3.6. Methylation analysis of fraction F-I

Original and carboxyl reduced fractions were methylated according to Ciucanu and Kerek (1984). The procedure was repeated until no absorbance was detected by I.R. at 2500–3500 cm^{-1}. The per-*O*-methylated polysaccharides were refluxed with 3% methanol-HCl for 3 h and then hydrolysed with 1 M H_2SO_4 at 100 °C for 18 h. The solutions were neutralized with $BaCO_3$ and the alditol acetates of partially methylated sugars analysed by GC–MS on DB-210 and OV-225 capillary columns (0.25 mm i.d.×30 m) linked to an ion trap, MS operated at 70 eV. The columns were programmed from 50 to 220 °C at 40 °C min^{-1}.

3.7. Biological experiments

C. graminicola was cultured in oatmeal-agar, under constant fluorescent light (60 $nE^{\mu-2,s-1}$) at 25 °C. Conidia (14 days old) were harvested, suspended in distilled H_2O and centrifuged (7200 *g* 2 min). The supernatant was removed and conidia re-suspended in H_2O and again centrifuged. This procedure was repeated five times to remove the extracellular matrix released during conidia sporulation (Mercure et al., 1994b). The final conidial concentration was adjusted to 1×10^6 conidia/ml with distilled H_2O.

3.8. In vitro experiments

The experiments were carried out using polystyrene slides as host surface. The conidial suspension was at a concentration of 1.10^5 conidia ml^{-1}, in spots of 50 μl. The two polysaccharide fractions isolated from *C. speciosa* were tested: F-I (from seed coats) and E-I (from exudate) in solutions at final concentrations of 0.62 and 0.12 mg ml^{-1}. The conidial suspension or distilled water were used as controls. The preparations with a mixture of conidial suspension and the polysaccharides (six spots) were applied onto the artificial surface, and incubated at 25 °C for 12 h. The surfaces were washed with distilled water, observed by light microscopy and photographed. The conidia count was carried out using a Neubauer's chamber and submitted to statistical evaluation (variance analysis and Student Test).

3.9. In vivo experiments

The experiments were carried out with a *Z. mays* plant, sample 41S (IAPAR). The seeds were placed onto a germination paper at 25 °C, with an 8-h photoperiod for 7 days. A mixture with the conidial suspension and F-I polysaccharide solution (2.5 mg ml^{-1}) was applied to the plant sheet surface. The plant remained for 8 days in a humidity chamber after application of the mixture (10 μl) on to the sheet upper surface. It was then observed and photographed. Only a conidial suspension or distilled H_2O water were used as controls. A second set of experiments was performed where drops (10 μl) containing the polysaccharide solution (2.5 mg ml^{-1}) were applied onto the sheet surface and dried. A conidial suspension was applied onto the dry polysaccharide in the same location. The plant was mantained in a humidity chamber for 8 days.

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