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Properties of the Seed Gum of *Stryphnodendron barbatiman* (barbatimäo)

Scientific Note

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INTRODUCTION

Watersoluble polysaccharides are finding a steadily widening range of industrial uses, notably as additives in food, pharmaceuticals, cosmetics, paper products, paints and plastics, and in oil well drilling muds, mining, and explosives. Their utility is based on functional characteristics. Before polysaccharides can be optimally exploited by industry, it is necessary to develop rules to predict their structure–function relationships. The effect of monosaccharide composition, configuration, and position of glycosidic linkages, type of sugar side chain, level of substitution and distribution of substituents along the main chain, conformation, and physical properties must be analyzed. Thus, the galactomannans, which are found as endosperm cell wall storage polysaccharides in many leguminous seeds, exhibit a wide range of novel and commercially useful rheological properties. In simple aqueous systems, they are effective viscosifiers, a property that is essentially controlled by molecular size (*1*) with the level

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of galactose substituents playing little or no role. Galactomannans can produce gels as the sole macromolecular component (2,3), or when associated with carragenans, agars, and xanthan (4,5). In contrast with the viscosant property, the level and distribution of galactose substitution along the mannan main chain play an important role in gel formation. Thus, the enzymatic action of α-D-galatosidase on a highly substituted galactomannan could lead to an increase in functionality if steps are taken to avoid main-chain cleavage by β-D-mannase impurities.

The uses of galactomannans and their presence in a wide variety of Brazilian trees directed our attention to new sources, such as *Mimosa scabrella* and *Stryphnodendron barbatiman*. The former whole seeds furnished approx 30% of galactomannan, which has a high galactose content (6). The rheological properties of its aqueous solution showed the typical behavior of a liquid-like solution, in contrast to most of the galactomannans described in the literature (7). *S. barbatiman* seeds also contained 30% of galactomannan, and its mannose to galactose ratio and $[\eta]$ are similar to those of guar gum (i.e., 1.5–1.0 and 1400 mL/g), respectively (8,9). It was observed that its useful rheology was dependent on the extraction process employed. When the benzene-ethanol pretreatment was used prior to aqueous extraction, the product was not viscous. In contrast, aqueous methanol pretreatment, followed by aqueous extraction at room temperature, afforded very viscous galactomannan solutions. Since most studies on polysaccharide properties are performed with commercial samples (10,11), the aim of the present investigation is to correlate methods of polysaccharide preparation from *S. barbatiman* seeds with the more interesting property of viscous solution formation.

MATERIALS AND METHODS

Polysaccharide Isolation

Crushed seeds (100 g) were twice refluxed with (4:1, v/v) methanol-water (4-h cycle). Insoluble material was then extracted with water (2 L) at room temperature with mechanical stirring for 24 h. The extract was treated with ethanol (4 L), and the precipitated polysaccharide (F-1) collected. Many variations were carried out for this preparation, as described below.

Pretreatment Variations

1. Reflux with the aqueous methanol water was carried out for 15, 30, and 60 min. A room temperature run for 3 h was used as a control.
2. Methanol-water ratios of 3:1 and 2:1 were used with 15 min of reflux.

3. A less polar extractant of ethanol:water (4:1, v/v) was used under reflux with 15 and 60 min of pretreatment.
4. Whole seeds were treated with boiling water for 15 min dried, and then crushed.

In all these pretreatments, polysaccharide extraction was carried out with water at room temperature (25°C) for 3 h, followed by ethanol precipitation.

Omission of Pretreatment

Individual lots of crushed seeds were submitted to direct aqueous extraction for 3 h at room temperature (F-2), and at 45, 55, 65, or 100°C. For the latter extractions, time was also shortened to 1 h.

Determination of Monosaccharide Composition

Polysaccharides were hydrolyzed with 1*M* trifluoroacetic acid at 100°C for 5 h, and the resulting monosaccharides converted into alditol acetates (*12*). Gas-liquid chromatography (glc) was performed at 190°C with a 3% OV-225 column (0.15 cm id × 200 cm on a Gas Chrom support; from Supelco Inc., Bellefonte, PA) with nitrogen as carrier gas (40 mL/min) and an FID detector.

Viscosity Measurements

Viscosity analyses were carried out using an Ostwald viscosimeter at 25°C. Definitions adopted were as follows:

$$\text{Specific viscosity} = [\eta_{sp}] = (t - t_o) / t_o \quad (1)$$

$$\text{Reduced viscosity} = 1 / c \cdot t - t_o / t_o \quad (2)$$

Intrinsic viscosity = $[\eta]$ = value expressed as L/g or 10^3 mL/g when plotting the reduced viscosities values against polysaccharide concentrations, where t = eflux time for the sample solution, t_o = eflux time for the solvent (water), and c = polysaccharide concentration in mg/mL.

Galactomannan and Xanthan Interaction

Solutions having a total concentration of 0.1% were prepared by mixing F-1 and xanthan solutions in order to obtain final component ratios of 4:1, 3:1, 2:1, 1:1, 1:2, 1:3, and 1:4.

^{13}C-nmr Spectroscopy

The spectrum was recorded at 30°C and 75 mHz with a Brucker AC-300 spectrometer in the Fourier transform mode. The F-1 sample was dissolved in D_2O in a 0.5-cm diameter tube. Chemical shifts were expressed as δ in ppm.

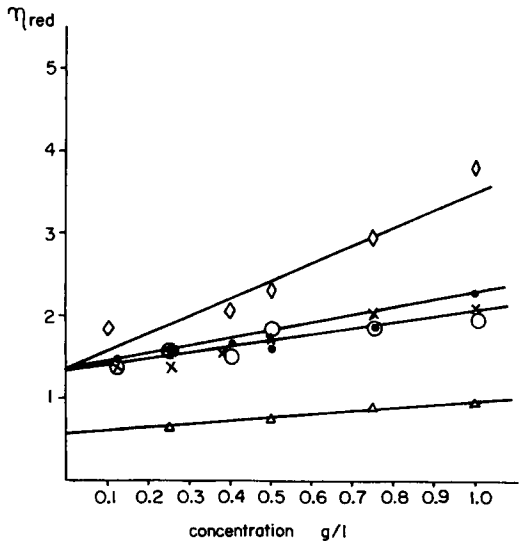


Fig. 1. Determination of intrinsic viscosities of galactomannan fractions of *S. barbatiman* prepared with 4:1 methanol:water pretreatment at different refluxing times. (Refluxing time: —◇— = 8 h; —●— = 1 h; —○— = 30 min; —×— = 15 min.; room temperature stirring for 3 h = —△—), ($[\eta]$ = L/g = 10^3 mL/g).

Determination of β-D-Endo-Mannanase Activity

Native, solvent-treated galactomannan (F-1, 0.1 g) was dissolved in water (100 mL). After complete dissolution, crushed seeds (0.5 g) of *S. barbatiman,* without pretreatment, were added. The mixture was kept at room temperature with mechanical stirring for 8 h and filtered. Polysaccharide(s) was precipitated with excess ethanol and analyzed by gel filtration chromatography as described previously (*8*). Its viscosity was then determined as described above.

RESULTS AND DISCUSSION

It was found that crushed seeds from *S. barbatiman* pretreated with methanol-water (4:1, v/v) under reflux for two cycles (4 h each) produced upon further water extraction at room temperature a viscous galactomannan solution ($[\eta]$ = 1400 mL/g). However, replacement of the refluxing solution by benzene-ethanol (9:1 v/v, Soxhlet, 8 h) or its omission yielded nonviscous galactomannan solutions from the aqueous extractions (*8*). The methanol-water under reflux step was proposed in order to remove low-mol-wt components (*13*). Since, in our experiments, a clear correlation between reflux pretreatment and polysaccharide viscosity was established, modifications in the pretreatment step were devised. Reflux with 4:1 methanol-water in isolated experiments, with progressive shortening

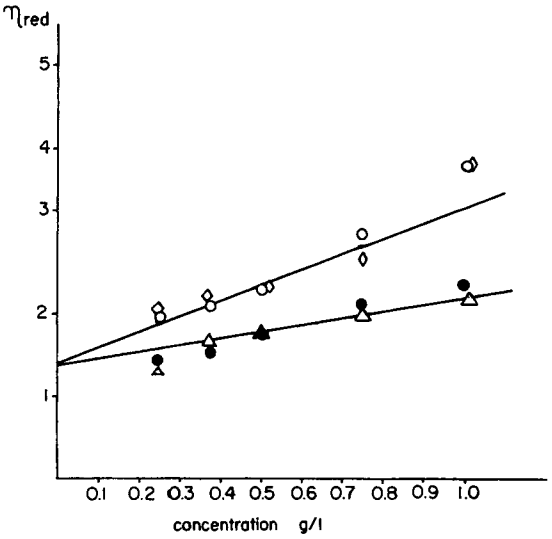


Fig. 2. Determination of intrinsic viscosities of galactomannans fractions of *S. barbatiman* prepared with different ratios of methanol:water as refluxing pretreatment. (—○—)=4:1 methanol:water, for 8 h; (—△—)=4:1, for 15 min; (—●—)=3:1, for 15 min; (—◇—)=2:1, for 15 min. (Experimental conditions as described in the text).

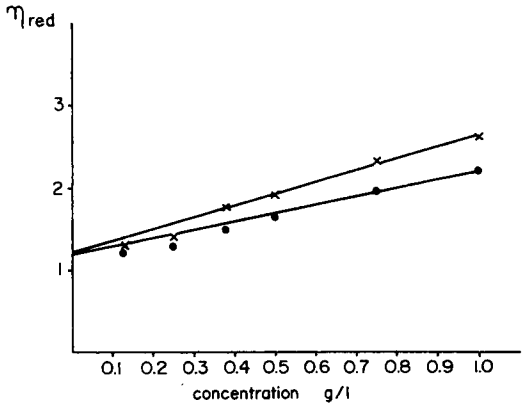


Fig. 3. Determination of intrinsic viscosities of galactomannan fractions of *S. barbatiman* prepared with 4:1 ethanol:water pretreatment at refluxing times of 60 min (—×—) and 15 min (—●—).

of the extraction period and maintenance of the subsequent water extraction for 3 h, was carried out. No alteration of the mannose to galactose ratio (1.5:1.0) of the galactomannan was found, and the highest value (i.e., $[\eta]$= 1400 mL/g) was observed, even in the shortest refluxing time of 15 min (Fig. 1). The same result was obtained by changing the reflux methanol-water mixture to 3:1 or 2:1 (Fig. 2) or rendering it less polar with ethanol as a substitute for methanol (Fig. 3). Despite the similar intrinsic viscosity

values, slopes of the latter plots of reduced viscosities against polysaccharide concentrations were not the same. Data curve superposition was found for the 4:1 (8 h) and 2:1 (15 min) methanol-water mixtures. Using the Huggins equation

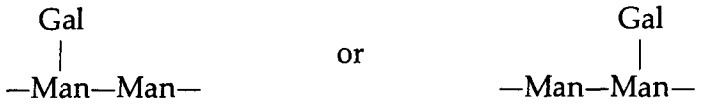
$$\eta_{sp/c} = [\eta] + k'[\eta]^2 c \quad (3)$$

where $\eta_{sp/c}$ is the reduced viscosity at concentration, c, the Huggins constant, k', was determined for each curve (*14*). This constant decreased from 1.19 to 0.61 and then to 0.36 in Fig. 1, from 0.91 to 0.41 in Fig. 2, and 0.76 to 0.64 in Fig. 3, considering the convergent curves. Generally, k' ranges from 0.3 to 0.7. Higher values are indicative of polymer association. Thus, the better galactomannan solution was obtained when seeds were pretreated with refluxing 4:1 methanol-water mixture during 15 min (k' = 0.36, Fig. 1). However, when methanol-water pretreatment was performed at room temperature (3 h) followed by the usual water extraction, the intrinsic viscosity of the galactomannan solution dropped to 600 mL/g (Fig. 1). This result clearly indicates that the temperature of the organic solvent pretreatment step is significant in addition to the composition of the extractant. In light of this observation, another set of experiments with temperature as variable was performed and correlated with the behavior of the resulting galactomannans. Crushed seeds were extracted individually and directly with water at temperatures of 45, 55, 65, and 100°C for 3 h. Again, the mannose to galactose polysaccharide ratio of 1.5:1.0 remained unchanged. However, the intrinsic viscosities found for these polysaccharides were proportionally affected (i.e., 30, 220, 550, and 900 mL/g, respectively). It is also noteworthy that the intrinsic viscosity of the galactomannan fraction obtained at 45°C was almost the same as that obtained at room temperature ($[\eta] = 27$ mL/g), and that the maximum $[\eta]$ value at 100°C (i.e., 900 mL/g) did not reach the value of the F-1 galactomannan resulting from the optimum reflux-based process (i.e., 1400 mL/g). However, when whole seeds were previously boiled in water and then crushed and extracted with water at room temperature, the viscosity of the product was again high (i.e., 1400 mL/g). The Huggins constant (k') from this plot was 0.4. These results are thus indicative of the occurrence of an endogenous galactomannan depolymerizing enzyme, probably a β-D-endo-mannanase, which can be progressively denatured on temperature increases or completely inactivated on reflux with methanol- or ethanol-based mixtures. Its complete denaturation through the brief boiling of the whole seeds in water reinforces this interpretation of the data. In the particular case of refluxing with benzene-ethanol (unexpectedly resulting in a nonviscous galactomannan solution), an incomplete contact between the pure organic mixture and the compact seed fragments could have occurred.

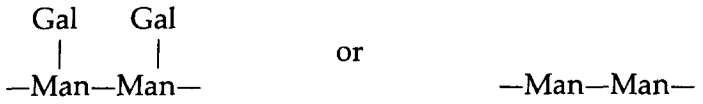
The seed galactomannan reserves are generally mobilized during the germination process and require, at least, three enzymatic activities, namely

α-D-galactosidase, β-D-endomannanase, and β-D-mannosidase (*15*). The galactosidase has been detected in leguminous tree seeds, as well as in other species, and its function is the cleavage of the unit sidechains of α-D-galactopyranose. Decreasing the galactomannan branching frequency does not implicate viscosity (*16*). The β-D-mannosidases of the endosperm show low activity (*15*), since they are active only against mannooligosaccharides and these are absent before seed germination. In relation to the native structure and rheological properties of galactomannans, endosperm β-D-mannanases are by far the more important. Most of these are endo-acting enzymes, as evidenced by the rapid fall in viscosity caused when acting on native polymeric substrates (*17*). The effectiveness of mannanases that hydrolyze galactomannans is related to the ratio of mannose to galactose in the polysaccharide, higher values obviously facilitating hydrolysis. Enzyme activity can be monitored by the increase in the reducing power, with the use of specific substrates, such as dyed galactomannans (*18*) or by procedures based on the viscosity decrease (*19*).

Thus, in correlating the viscosity loss of the *S. barbatiman* galactomannan extracted directly with water at room temperature with the 83% decrease if the mol wt as determined by gel filtration, the action of a β-D-endomannanase is suggested. This was not detected using reducing sugar estimations because of the large mol wt of F-1 or its degraded product (F-2). Since no free D-galactose was found and its content in the native polysaccharide accounts for 40%, there are only a few points susceptible to mannanase attack. It requires two or more unsubstituted mannosyl units (*20*), but as indicated by the ^{13}C-NMR spectrum (Fig. 4), the main mannose diad in the *S. barbatiman* galactomannan is



determined by C4-mannosyl signals at 77.73 ppm. The intensities of the signals for the diads



at 77.90 and 77.43 ppm, respectively (*20–22*), are slighter. The native polysaccharide, accordingly, did not interact with xanthan gum in all the proportions examined. This type of interaction is also dependent on nonsubstituted regions in the mannan backbone (*1,5*).

In a further experiment, a solution of F-1 ($[\eta] = 1400$ mL/g) was treated with crushed and unpretreated seeds. The mixture was stirred at room temperature during 8 h and filtered. Polysaccharide was then isolated, and its intrinsic viscosity determined to be 140 mL/g, thus suggesting β-D-endo-mannanase activity arising from the added seeds. This same poly-

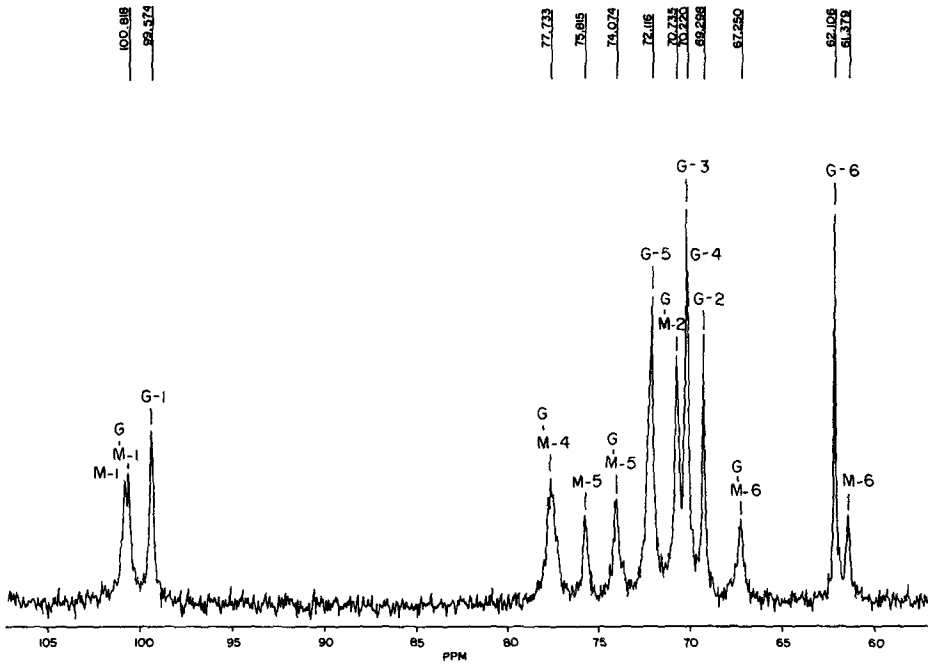


Fig. 4. ^{13}C-NMR spectrum of the galactomannan from seeds of *Stryphnodendron barbatiman* in D_2O at 30°C and 75 mHz. (M= β-D-mannopyranosyl units; G=α-D-galactopyranosyl units).

saccharide sample was subjected to gel permeation chromatography on a Sepharose CL-4B column and eluted as a single peak. The V_e of this peak was 79 mL, similar to that of F-2 (V_e=82 mL), whereas the V_e of F-1 was 54 mL. The empirical equation of Mark-Khun-Houwink $[\eta]=k\cdot M_w^{\alpha}$ relates intrinsic viscosity to mol wt for a neutral polymer (*23,24*). Then, one possible explanation for the above results is a decrease in galactomannan mol wt upon the action of a seed β-D-mannanase.

CONCLUSIONS

Pretreatment resulting in enzymatic inactivation proved to be essential for the preparation of highly viscous solutions of the galactomannan from seeds of *S. barbatiman* ([η]=1400 mL/g). Short periods of reflux with aqueous methanol or ethanol or treatment with boiling water of the seeds was used. The target for the inactivation step is a β-D-endo-mannanase activity present in the dormant seeds.

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