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Characterization of the Galactomannans from *Parkinsonia Aculeata* Seeds and their Application on Affinity Chromatography

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Abstract: Successive aqueous (10 °C, 25 ºC and 80 °C) and alkaline (1M NaOH; 25 °C) extractions of milled *Parkinsonia aculeata* endosperms gave rise to four galactomannan fractions. These extractions furnished viscous galactomannans with Man:Gal ratios ranging from 3.1:1; 3.7:1; 4.9:1 and 6.1:1 (P1, P2, P3 and P4, respectively). Fraction P1 was used for structural studies by using methylation analysis, periodate oxidation and ^{13}C-NMR. It showed a linear backbone of $\beta(1\rightarrow4)$ linked D-mannose units, to which single $\alpha(1\rightarrow6)$-linked D-galactose are attached. This galactomannan has Mw 775700 g/mol and intrinsic viscosity of 558 mL/g. The four fractions and the crude endosperm were treated with epichlorydrin and used as matrix for affinity chromatography. All columns tested showed ability to bind lectin samples. The efficiency is related to the degree and pattern of substitution of galactosyl units on the D-mannan backbone.

Keywords: Galactomannan*, Parkinsonia aculeata,* lectin, affinity chromatography.

Introduction

Galactomannans are neutral polysaccharides obtained from the seeds of some *Leguminosae*. They are found as endosperm cell wall storage compounds, having a linear backbone of $\beta(1 \rightarrow 4)$ linked D-mannopyranosyl units, to which single α-D-galactopyranosyl units are attached at O-6 (between 5% and 100%). The main difference between galactomannans from different plant sources lies in the galactose content as well in its distribution along the mannopyranosyl backbone[1,2].

Their solution properties and applications are related both to the structural features and the molecular masses. The fine structures of galactomannans have been studied by analysis of oligosaccharides formed on partial enzymatic[3,4] and acid hydrolysis[5,6]. Experimental and theoretical approaches have considered the conformation features of galactomannans. It has been shown that the unperturbed dimensions are affected by the degree and pattern of substitution[7,8].

Galactomannans from *Ceratonia siliqua* (locust bean gum), *Cyamopsis tetragonolobus* (guar gum) and *Caesalpinia spinosa* (tara gum), with Man:Gal ratios of 4:1; 2:1 and 3:1, respectively, are commercially available. These hydrocolloids are widely employed in the food, pharmaceutical and cosmetic industries as thickening and stabilizing agents[1,2]. Galactomannans have also been tested as a sieving matrix in capillary electrophoresis[9]. In addition, chemically modified galactomannans display unique features in novel applications, such as crosslinked galactomannans[10]. The crosslinked hydrogels are gaining much importance in a wide variety of applications as drug carriers or in affinity chromatography. Moreira et al[11] isolated a lectin from the saline extract of *Artocarpus incisa* (fruta pão) seed by affinity chromatography on crosslinked *Adenanthera pavonina* (carolina) galactomannan.

In the present investigation galactomannans from *Parkinsonia aculeata* (turco) were characterized and used to prepare affinity chromatography columns for lectin isolation.

Experimental

Materials: seeds of *Parkinsonia aculeata, Artocarpus integrifolia*, *Artocarpus incisa* and *Abrus precatorius* were collected in the State of Ceará (Brazil). Rabbit blood cells were obtained by puncture of the ear's marginal vein of healthy animals.

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Polysaccharide isolation: whole seeds of *Parkinsonia aculeata* were boiled in H_2O for 10 min and then kept at 4 ºC for 24h until swelling took place. Thereafter, the endosperms were separated manually. The dry endosperm was milled and submitted to successive aqueous extractions (10 ºC, 25 ºC and 80 ºC) and then alkaline extraction (1M NaOH; 25 ºC). Polysaccharides were obtained from each extract by precipitation with ethanol (2 vol).

Chemical analysis: total carbohydrate was estimated by phenol-sulphuric acid[12], total nitrogen by Baethgen & Alley[13], and soluble protein by the Hartree[14] or Bradford[15] method.

Polysaccharide characterization: the biopolymers were hydrolyzed with 1M trifluoroacetic acid solution (5 h, 100 ºC), the hydrolyzates evaporated, the residues reduced with $NaBH_4$, and the products acetylated with pyridine-acetic anhydride (1:1 v/v, 16 h, at 25 ºC). The resulting alditol acetates were analyzed by GLC (gas-liquid chromatography) using a model 5890 S II HP Gas Chromatograph at 220 ºC (FID and injector temperature, 250 ºC) with a DB-210 capillary column (0.25 mm i.d. x 30m), film thickness 0.25 µm, the carrier gas being nitrogen.

Paper chromatography (PC) was conducted by the ascending method, on Whatman nº 1 paper using as solvent the system benzene-1-butanol-pyridine-water (1:5:3:3 v/v; upper phase). The sugar was detected by alkaline $AgNO_3$[16].

^{13}C-NMR spectra (75MHz) were recorded at 80 ºC and 75 MHz with a Bruker AC-300 spectrometer. The samples were dissolved in 1 M NaOH/D_2O.

Homogeneity and molecular weight were determined by HPSEC-MALLS (high performance size exclusion chromatography - multi angles laser light scattering) using 0.1 M $NaNO_2$ as eluent.

The viscosity determination was performed in a Brookfield LDV-III rheometer, with a cone plate spindle (CP40 or SC4-18) coupled to a Brookfield TC-500 circulating bath that maintained the temperature at 20 ºC.

Methylation of galactomannan was performed according to Ciucanu & Kerek[17] and the per-*O*-methylated polysaccharide hydrolyzed and analyzed by GLC-MS (mass spectroscopy) using a 330 Varian instrument equipped with an OV-225 capillary column (0.25mm i.d. x 30m) linked to a Finnigan-MAT mass spectrometer.

A galactomannan sample was submitted to $NaIO_4$ oxidation at 25 ºC (2 cicles) and the products were analyzed by GLC of derivated alditol acetates as previously described[18].

Preparation of crosslinked galactomannans: the crosslinked polysaccharides were prepared as described by Appukuttan, Surolia & Bachhawat[19]. Conditions used were 0.5 g of polysaccharide, 0.5 mL epichlorydrin and 4.0 mL of 3M NaOH.

Haemagglutination activity: clumping of red blood cells by the various fractions obtained during purification was estimated as described before by Moreira & Perrone[20].

Lectin extraction: dehulled seeds of *Artocarpus integrifolia*, *Artocarpus incisa* and *Abrus precatorius* were dried with acetone, milled and stirred with 0.15M NaCl. The suspensions were left at room temperature for 2h and then centrifuged (9000.g; 20 min; 4 ºC). The clear supernatants were used for determination of protein content and haemagglutinating activity.

Lectins purification: the saline crude extracts were precipitated by $(NH_4)_2SO_4$ (0-80% of saturation), dialysed against 0.15 M NaCl and applied to *Parkinsonia aculeata* cross-linked galactomannans column equilibrated with the same solution. After elution of the non-retained fraction, the lectin was eluted with pH 2.6, 0.1 M Glycine-HCl buffer, containing 0.1 M NaCl or 0.2 M galactose containing 0.15 M NaCl. The clear supernatants were used for determining the protein content and haemagglutinating activity.

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE): it was carried out as proposed by Laemmli[21]. Samples of lectins were dissolved in 0.01 M phosphate buffer, pH 7.0, 2 % SDS with 1 % b-mercaptoethanol and incubated at 100 ºC for 15 min. A few crystals of sucrose were dissolved in the samples which were then applied to the gel (17.6% polyacrylamide and 4% stacking gel). The electrophoresis was conducted at a constant current of 13 mA for 4h. The protein bands were visualized by staining with Coomassie Brilliant Blue R-250.

Results and Discussion

The protein content in the crude endosperm of *Parkinsonia aculeata* was estimated to be 4.5 % by using the total nitrogen results.

Successive aqueous (10 ºC, 25 ºC and 80 ºC) and alkaline (1M NaOH; 25 ºC) extractions of milled endosperm gave rise to polysaccharide fractions 1, 2, 3 and 4, respectively. These extractions furnished viscous galactomannans with Man:Gal ratios ranging from 3.1:1 to 6.1:1 (Table 1). Seeds from other *Leguminosae* species have been described to contain a system of galactomannans with different levels of galactose substitution[18,22]. However, our results contrast with those obtained by Gurha and Singh[23], who studied the polysaccharides from the seeds of *Parkinsonia aculeata* and described only a galactomannan with Man:Gal ratio 1.85:1.

The largest polysaccharide yield (26.2% w/w, based on dry endosperm) occurred in the first water extraction, and this was used for structural studies. Fractions P1 and P4 showed the presence of pentoses as minor components. They are probably products of contaminating hemicelluloses.

Results from methylation analysis of fraction P1 showed 2,3,6-Me_3-Man as the main derivative (58.0%). The 2,3,4,6-Me_4-Man (5.9%) corresponds to the non reducing mannosyl ends. Similar amounts of the 2,3-Me_2-Man and 2,3,4,6-Me_4-Gal were found. These results, which agree with the periodate data are consistent with a main chain of (1→4)-linked mannopyranosyl units substituted at O-6 with galactopyranosyl units, in spite of an unusual component, the 2,4,6-Me_3-Gal, was also found as minor component. This

Table 1. Yields and monosaccharide composition of polysaccharides obtained from the endosperm of *Parkinsonia aculeata*.

Fraction	Extractant	Yield[a]	Monosaccharide composition[b] (%)				
		(%)	Ara	Xyl	Man	Gal	Man:Gal ratio
P1	Water, 10 ºC	26.2	1.4	0.6	73.9	24.1	3.1:1
P2	Water, 25 ºC	7.9	—	—	78.8	21.2	3.7:1
P3	Water, 80 ºC	17.9	—	—	83.3	16.7	4.9:1
P4	1M NaOH, 25 ºC	2.9	10.9	—	76.5	12.6	6.1:1

[a] Based on dry endosperm
[b] Determined by GLC

Table 2. ^{13}C NMR chemical shifts for the galactomannan P1 from *Parkinsonia aculeata* endosperm.

Unit	C-1	C-2	C-3	C-4	C-5	C-6
α-D-galactopyranosyl	99.10	68.78	70.25	69.67	71.48	61.41
β-D-mannopyranosyl unbranched	100.31	70.44	71.80	76.42	75.35	60.84
β-D-mannopyranosyl branched	100.31	70.44	71.80	77.00	75.59	66.75

could be due to the presence of O-3 substituted galactopyranosyl units, however results from periodate oxidation showed glycerol, erythritol and only traces of galactose, which indicates that no significant number of (1→3)-linkages are present, suggesting uncompleted methylation.

On the other hand, the ^{13}C-NMR spectrum of the galactomannan P1 is in close agreement with those reported for other conventional galactomannans[18,24]. Three types of structural units were identified, namely α-D-galactopyranosyl units, unsubstituted (1→ 4)-linked β-D-mannopyranosyl units of the mannan main chain and the O-6 substituted (1→ 4)-linked β-D-mannopyranosyl units. Chemical shifts are recorded in Table 2. No assignment for 1→ 3 linkage was observed.

The Mw for galactomannan P1, determined by HPSEC-MALLS is 775700 g/mol, with Mw/Mn of 2.84, indicating polydispersity, that could arises from aggregation. Its intrinsic viscosity was determined in the Newtonian regime as 558 mL/g using water as solvent (Figure 1). This value is lower than those obtained for other galactomannans from Brazilian seeds[5] and the others commercialized[1,2]. However, this characteristic could make easier the crosslink reaction when compared to high viscous polymers.

The four galactomannan fractions and the crude endosperm obtained from the seeds of *Parkinsonia aculeata* were submitted to treatment with epichlorydrin. Different conditions of treatment were previously tested (galactomannan, epichlorydrin and NaOH)[25]. The best results were obtained by using 0.5 g of polysaccharide, 0.5 mL epichlorydrin and 4.0 mL of 3M NaOH, conditions which were used in this work. Galactomannans from guar (Sigma Chemical Co) and *Adenanthera pavonina* (Man:Gal ratio 1.8:1), previously characterized by Tavares[26], were also crosslinked under the same conditions. These crosslinked

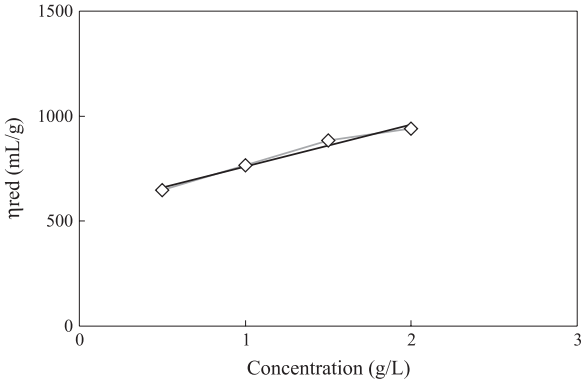

Figure 1. Determination of intrinsic viscosity of galactomannan P1 from *Parkinsonia aculeata* endosperm.

materials were used as matrix for affinity chromatography for lectins purification.

D-galactose specific lectin extracts from *Artocarpus integrifolia*, *Artocarpus incisa* and *Abrus precatorius* were applied to the crosslinked galactomannan (guar, *Adenanthera pavonina* and crude endosperm of *Parkinsonia aculeata*) columns (Figures 2-4) . After removing the unbounded protein (detected by UV 280 nm), the lectins were desorbed from the columns with 0.2 M galactose solution containing 0.15 M NaCl.

The crosslinked galactomannan from crude endosperm of *Parkinsonia aculeata* showed ability to bind lectins. Similar results were obtained when the lectin extracts were applied to *Adenanthera pavonina* and guar crosslinked galactomannan columns. All columns showed lectin binding properties. The guar galactomannan column was the most efficient, followed by *Adenanthera pavonina* and then the crude endosperm of *Parkinsonia aculeata.* On the other hand, *Abrus precatorius* lectin was not efficiently isolated in the three columns (Table 3).

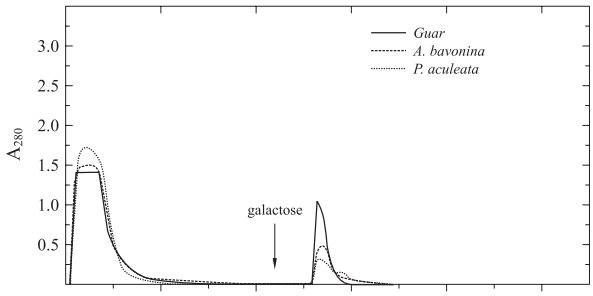

Figure 2. Affinity chromatography of *Abrus precatorius* seed crude extract on guar, *Adenanthera pavonina*, *Parkinsonia aculeata* crosslinked galactomannans.

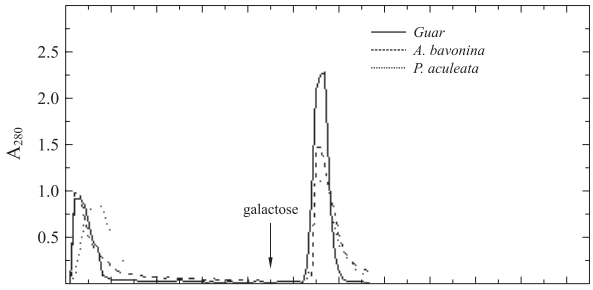

Figure 3. Affinity chromatography of *Artocarpus integrifolia* seed crude extract on guar, *Adenanthera pavonina*, *Parkinsonia aculeata* crosslinked galactomannans.

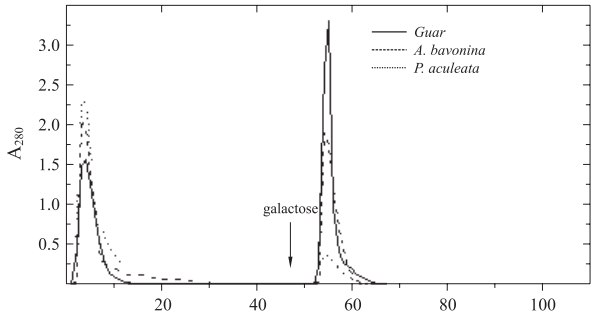

Figure 4. Affinity chromatography of *Artocarpus incisa* seed crude extract on guar, *Adenanthera pavonina*, *Parkinsonia aculeata* crosslinked galactomannans.

Table 3. Protein fractionation by affinity chromatography on *Parkinsonia aculeata* crosslinked galactomannan.

Crude Extract	Unbounded fraction (%)	Retained fraction (%)
Artocarpus incisa	85	15
Artocarpus integrifolia	84	16
Abrus precatorius	99	1

The ability of crosslinked galactomannans to isolate lectins was confirmed by SDS PAGE electrophoresis (Figure 5). The fractions eluted with the galactose solution from affinity columns had only the bands corresponding to the lectins and showed hemagglutinating properties[11].

Moreover, the efficiency to bind lectin samples were compared, using the *Parkinsonia aculeata* crosslinked galactomannan columns prepared with fractions P1 to P4 (Figures 6-8). The ability of these galctomannans to bind lectins was related with the degree of substitution. The better results were obtained with fraction P1, the galactomannan more substituted by galactose, which Man:Gal ratio is 3.1:1.

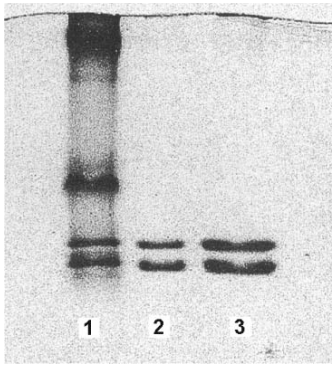

Figure 5. SDS-polyacrylamide gel electrophoresis in the presence of 2-mercaptoethanol of 1) seed crude extract from *Artocarpus incisa*, 2) purified lectins from *Artocarpus incisa* and 3) *Artocarpus integrifolia* isolated by affinity chromatography on *Parkinsonia aculeata* crosslinked galactomannan.

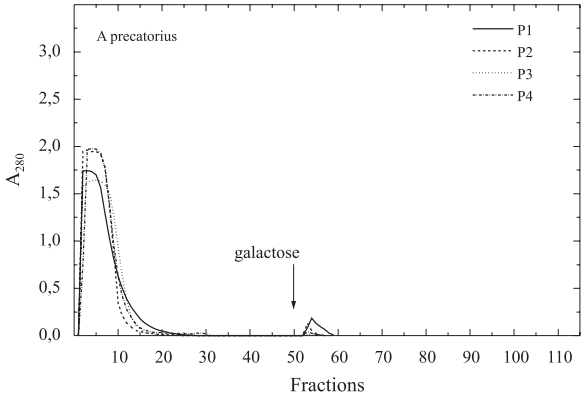

Figure 6. Affinity chromatography of *Abrus precatorius* seed crude extract on *Parkinsonia aculeata* crosslinked galactomannan fractions P1 to P4.

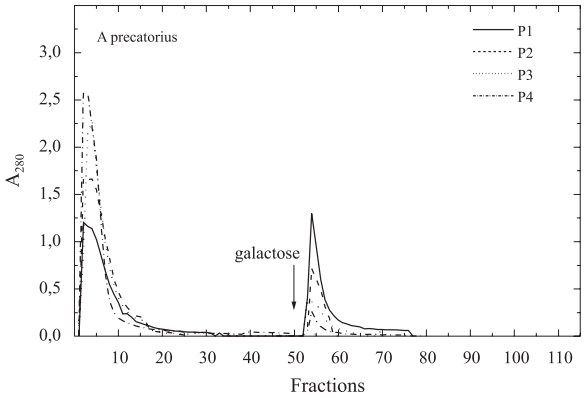

Figure 7. Affinity chromatography of *Artocarpus integrifolia* seed crude extract on *Parkinsonia aculeata* crosslinked galactomannan fractions P1 to P4.

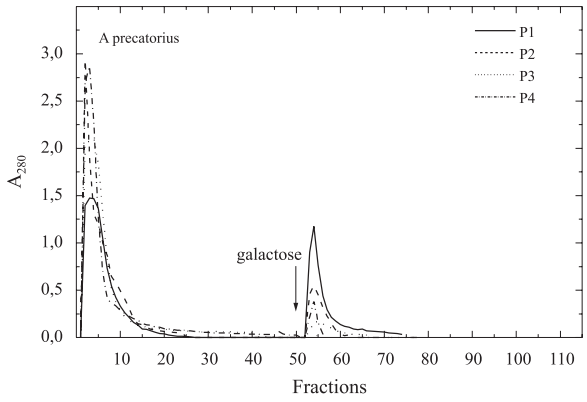

Figure 8. Affinity chromatography of *Artocarpus incisa* seed crude extract on *Parkinsonia aculeata* crosslinked galactomannan fractions P1 to P4.

The different efficiencies between the *Parkinsonia aculeata* matrix and those prepared with guar and *Adenanthera pavonina*, which have similar Man:Gal ratios could be attributed to pattern of galactosyl distribution. Further studies will be performed with the highly substituted galactomannan from *Mimosa scabrella* (Man:Gal ratio 1.1:1)[27] and other such as *Stryphnodendron barbatiman* (Man:Gal ratio 1.5:1)[28].

Conclusion

Seed endosperms from *Parkinsonia aculeata* contain a system of galactomannans with different levels of galactose substitution, with Man:Gal ratio ranging from 3.1:1 to 6.1:1. Affinity chromatography columns were prepared with these fractions as well as guar galactomannan (Sigma Chemical Co) and that obtained from the seeds of *Adenanthera pavonina* crosslinked with epichlorydrin and showed to be efficient in the purification of D-galactose specific lectin samples. The efficiency is related to the degree and pattern of substitution of galactosyl units on the D-mannan backbone. These modified galactomannans hydrogels could, thus, be used to substitute the expensive commercial affinity chromatography gels.

Acknowledgments

PRONEX, PROCAD-UFPR/UFC.

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