STRUCTURAL DETERMINATION AND CHEMICAL MODIFICATIONS OF THE POLYSACCHARIDE FROM SEEDS OF *Prosopis chilensis* Mol. (Stuntz)

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ABSTRACT

The polysaccharide extracted from *Prosopis chilensis* seeds in acidic medium showed by total hydrolysis and gas-liquid chromatography analysis of the alditol acetates to be composed of galactose and mannose in the molar ratio 1.0:1.9. Studies by methylation and ^{1}H and ^{13}C NMR spectroscopy indicated that the polysaccharide was a galactomannan with a chain of D-mannopyranosyl residues linked β 1 $\rightarrow$ 4 which carried alternatively α-D-galactopyranosyl residues at position *O*-6 of D-mannose units. The chemical modification of the galactomannan by reaction with sodium chloroacetate afforded in 95.3% yield, a derivative which gave a very viscous solution with water. By titration it was determined a carboxymethylation degree of 0.42 indicating that 64% of the alcoholic groups were etherified. Reaction of the galactomannan with TEMPO-NaCl-NaOBr system gave a water soluble polysaccharide. The DEPT 135º NMR spectrum indicated that the primary alcoholic groups of galactopyranosyl and unbranched mannopyranosyl residues were fully oxidised.

Key words: *Prosopis chilensis,* seeds, polysaccharide, galactomannan, carboxymethylation, TEMPO oxidation.

INTRODUCTION

Mesquite is the common name applied to several *Prosopis* species, which have been used for many years as a source of food, fodder and fuel[1]. *Prosopis juliflora* is native from Central America, it exudates a polysaccharide known as mesquite gum similar to gum arabic (1→3 β-D-galactopyranan branched at position *O*-6)[2,3]. The endosperm of the seeds of *Prosopis* species contains a polysaccharide similar to locust bean and guar gums. The latter is a galactomannan extracted from seeds of *Cyamopsis tetragonoloba,* it contains a linear chain of β-D-mannopyranosyl residues linked 1→4, alternatively substituted by α-D-galactopyranosyl residues at position *O*-6[4]. The polysaccharide from seeds of *Prosopis juliflora* collected in the southwest of United States contains galactose and mannose in the molar ratio of 1:2, while the galactomannan from seeds of Brazilian *P*. *juliflora* is rich in mannose residue (galactose: mannose ratio 1:4)[1].

Chemical modifications of polysaccharides allow the preparation of new polymers with specific properties[5]. By etherification of the alcoholic groups of cellulose and starch, derivatives with industrial applications were obtained[6]. A method for the selective oxidation of primary alcoholic groups in water-soluble polysaccharides with sodium hypochlorite, sodium bromide and catalytic amounts of 2,2,6,6-tetramethyl-1-piperidine oxoammonium salt (TEMPO) was developed by De Nooy *et al*.[7-8]. Oxidation of native samples of cellulose with this system allowed the introduction of small amounts of carboxyl groups[9]. Recently, Tahiri and Vignon[10] obtained polyglucuronans, soluble in water, by applying the TEMPO-NaBr-NaClO system to amorphous cellulose samples.

Prosopis chilensis (Leguminosae) grows in the semi-arid regions of Perú, Northern Chile and Argentina.[11] According to Escobar *et al.*[12] the seeds contain ~ 30 % of a viscous gum.

The aim of this work was to elucidate the structure of the polysaccharide from *Prosopis chilensis* seeds and to study the chemical modification to improve the solubility in water.

EXPERIMENTAL

Materials and methods

The preparation of the acidic extract by treatment of *Prosopis chilensis* seeds with 70% H_2SO_4 was previously described[13]. Total sugars were determined by the phenol-sulfuric acid method using D-mannose as standard[14]. The content of uronic acids was assayed according to Filisetti-Cozzi and Carpita[15] using D-galacturonic acid as standard. Carbon and hydrogen microanalysis were performed in Facultad de Química, Universidad Católica de Chile. Gas-liquid chromatography (GLC) analysis of the alditol acetates was carried out in a Shimadzu GC-14B gas chromatograph equipped with a flame ionization detector using a fused silica capillary column (30 m x 0.25 mm) coated with SP-2330. GLC was performed with an initial hold at 150 ºC for 2 min and then at 5 ºC/min to 210 ºC for 10 min. The helium flow rate was 20mL/min and the detector and injector temperature was 220 ºC. The identities of all derivatives were determined by comparison with authentic standards. GLC-MS analysis were performed in Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Argentina, as previously described[16]. FT-IR spectra were obtained in KBr pellets according to the method described earlier[17]. ^{13}C NMR spectra were registered on a Bruker Avance DRTX 400 spectrometer operating at 100.62 MHz, at 70 ºC after isotopic exchange with

D_2O (3 x 0.75 mL) using D_2O as solvent with MeOH as internal reference ($d^{13}C$: 49.50 ppm). Molecular weight determination by the reducing end assay was performed as described by Cáceres et al.[18]. Absorbance was registered with a Genesys 5 double beam spectrophotometer.

Purification of the extract

The extract (1.0 g) and 200 mL of distilled water were stirred at 70 ºC for 36 h. The resulting solution was dialysed against distilled water for 48 h using 3500 cut-off membrane, concentrated in vacuo and poured on 5 times its volume of ethyl alcohol. The precipitate was separated by centrifugation (3000 x g) and dried in an oven at 50 ºC.

Total hydrolysis and monosaccharides analysis

The polysaccharide (0.020 g) was heated with 4 mL of 2 M TFA during 2 h at 120 ºC. The acid was removed in vacuo by repeated co-evaporations with distilled water. An aliquot of the resulting syrup was dissolved in a minimum amount of water and 0.002 g of sodium borohydride was added. The mixture was stirred 2.5 h at room temperature and then, treated with Zeo-Karb 225 resin until the pH of the solution decreased to 5.0. The filtrate was concentrated to dryness, treated with Ac_2O in anhydrous pyridine and analysed by GC. The rest of the hydrolysate was treated with (S)-1-amino-2-propanol in methyl alcohol in the presence of $NaBH_3CN$ according to Cases, Cerezo and Stortz[19]. The resulting syrup was acetylated with Ac_2O-pyridine (1:1 v/v) and analysed by GC-MS

Gel permeation chromatography

The polysaccharide (0.003 g) in 1 mL of water was chromatographed on a Sephadex G-200 column (100 x 1.5 cm) using 0.2 M NaCl as eluant. The column was calibrated with 0.3% Blue dextran 2000 and D-glucose solutions. Fractions of 3 mL were collected and elution was monitored spectrophotometrically with the phenol-H_2SO_4 reagent[14].

Partial hydrolysis

The polysaccharide (0.100 g) was stirred with 1.5 mL of concentrated HCl for 15 min. at room temperature and poured into 100 mL of acetone. The precipitate was washed thrice with portions of 2 mL of acetone, dissolved in water and freeze-dried.

Methylation

Methylation of the polysaccharide was performed according to Ciucanu and Kerek[20]. Briefly, the polysaccharide (0.015 g) in 3 mL of dimethylsulfoxide was stirred with powdered (0.150 g) NaOH for 2 h at room temperature. Then, methyl iodide (1.5 mL) was added and the mixture was stirred for 1 h. The addition was repeated twice and the reaction was stopped by addition of water (1 mL), dialysed against distilled water and freeze-dried. The resulting solid was hydrolysed as described earlier and analysed as alditol acetates by GLC-MS.

Carboxymethylation

The polysaccharide (0.300 g) in 5.0 mL of 2-propanol was stirred with 0.5 mL of 30% aqueous solution of NaOH for 1 h and chloroacetic acid (0.360 g) was added. The mixture was stirred at 55 ºC during 3.5 h and the solid was separated by centrifugation, washed

with 70% aqueous solution of MeOH and neutralised with 90% acetic acid. The resulting solid was dissolved in distilled water, dialysed against distilled water and freeze-dried. The modified polysaccharide (0.0214 g) was stirred with 2 mL of water, 0.5 mL of 70% MeOH and 0.5 mL of 0.4055 N NaOH for 4 h. The resulting solution was titrated with 0.440 N HCl using phenolphthalein as indicator. The degree of substitution was calculated according to Green using the formula: D.S. = 0.162A/(1-0.058A), where A = milliequivalents of NaOH required per gram of samples[21].

Oxidation with TEMPO

The polysaccharide (0.125 g) was suspended in 67 mL of distilled water and 0.001 g of TEMPO and 0.008 g of sodium bromide were added.. To the mixture at 3 ºC±1 ºC, 4.5 mL of 10% sodium hypochlorite was added and the pH was maintained at 9.45 by addition of a 0.05 M sodium hydroxide for 1.5 h. Then, $NaBH_4$ (0.050 g) was added, the pH adjusted at 8.0 with 2 M HCL and it was stirred for 1 h. The resulting solution was dialysed against tap water for 24 h and then, with distilled water, concentrated *in vacuo* and poured on 5 times its volume in EtOH. Uronic acid content was determined by the carbazol method[22].

RESULTS AND DISCUSSION

Purification of the acidic extract from *Prosopis chilensis* seeds afforded a white powder in 88.2 % yield. The elementary microanalysis (39.01 % carbon, 6.49 % hydrogen, 0.0 % nitrogen) showed the absence of proteins and a CH_2O formula. By spectrophotometric determination no uronic acid was detected, which indicates that the purified extract is a neutral polysaccharide. Total acid hydrolysis and GLC analysis of the derived alditol acetates showed the presence of galactose and mannose in the molar ratio 1:1.9. The ratio is very similar to that reported for guar gum and higher in mannose content than the galactomannan from *Prosopis africana* seeds[23]. GLC analysis of the 1-deoxy-1-(2-hydroxy-propilamino) alditol acetates derived from the total hydrolysis, reductive amination and acetylation product of polysaccharide (PSA) indicated that both monosacharides showed the D-configuration. Gel filtration chromatography on Sepharose CL-4B of PSA showed that it was homogeneous. Its molecular weight, determined by the end-reducing method was 1,167,400.

The ^{13}C NMR DEPT 135 spectrum (Fig. 1) showed at high field three inverted signals which were assigned to methylenic carbons of glycosyl residues. The presence of 18 signals indicated a quite regular repeating trisaccharide unit. Resonances were assigned with the aid of literature data[24,25] and are presented in Table 1.

The spectrum presented only one signal for C-6 of branched β-D-mannopyranosyl residue which may indicate according to Manzi *et al*.[26], the presence of a D-mannosyl triad where the intermediate residue is substituted.

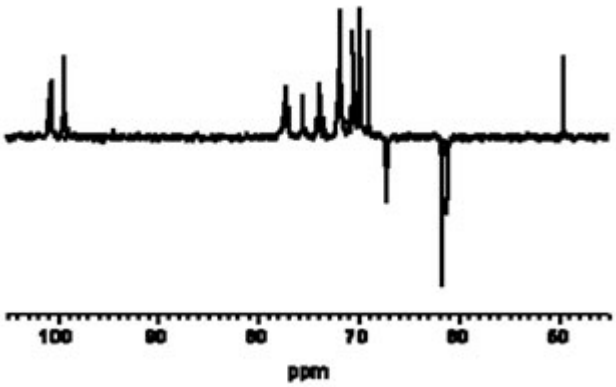

Figura 1. ^{13}C NMR DEPT 135º spectrum in D_2O of partially hydrolysed polysaccharide from *Prosopis chilensis* seeds.

Table 1. Assignments of the resonances of the ^{13}C NMR DEPT 135º spectrum of partially hydrolysed polysaccharide from *Prosopis chilensis* seeds.

Residue	Chemical shift (δ, ppm)					
	C-1	C-2	C-3	C-4	C-5	C-6
α-D-Galactopyranosyl	99.37	69.01	69.86	70.01	71.76	61.72
β-D-Mannopyranosyl	100.72	71.48	72.03	77.00 77.30	75.59	61.12
β-D-Mannopyranosyl branched at O-6	100.55	71.48	71.93	77.00 77.53	73.91	67.20

The polysacharide was submitted to two cycles of methylation, total hydrolysis and GLC-Ms analysis of the derived alditols acetates. Results are shown in Table 2.

Table 2. Methylation analysis of the polysaccharide from seeds of *Prosopis chilensis.*

Peracetate of partially methylated sugars	Fragments (m/z)	%	Molar ratio
2,3,4,6-Tetra-O-methyl-galactitol	43, 117, 161, 205	30.89	1.00
2,3,6-Tri-O-methyl-mannitol	43, 117, 161, 173, 233	24.81	0.82
2,3-Di-O-methyl-mannitol	117, 161, 201, 261	38.18	1.05
Galactitol		3.54	0.08
Mannitol		2.56	0.06

The presence of 2,3,4,6-tetra-*O*-methyl-galactitol acetate could be ascribed to terminal galactopyranosyl residues. The molar ratio of 1,4,5,6-tetra-*O*-acetyl-2,3-di-*O*-methyl-

mannitol is very similar to that of the galactopyranosyl derivative. The presence of 2,3,5-tri-O-methyl-mannitol acetate in similar amount allowed to conclude that one of two residues in 1□ 4 linked mannan carried a galactopyranosyl residue at position *O*-6.

The results obtained by methylation analysis of PSA corroborate those obtained by ^{13}C NMR spectroscopy and indicated that the polysaccharide extracted in acidic medium from seeds of *Prosopis chilensis* was a galactomannan with a chain of D-mannopyranosyl residues linked β 1□ 4 which carried alternatively α-D-galactopyranosyl residues at position *O*-6 of a mannose unit. The proposed structure is shown in figure 2.

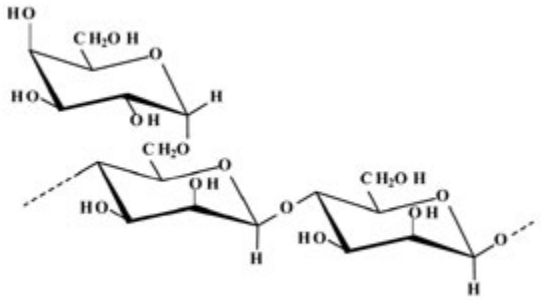

Fig. 2. Structure of the galactomannan from *Prosopis chilensis* seeds.

Carboxymethylation of native polysaccharide

Reaction of the native polysaccharide from *Prosopis chilensis* with monochloroacetic acid in alkali medium afforded in 95.3 % yield an amorphous solid which gave very viscous solutions in water. The FT-IR spectrum of the reaction product presented a signal at 1605 cm^{-1} assigned to the stretching vibration of carboxylate group. The ^{13}C NMR spectrum showed signals at 178.32 ppm assigned to the carbonyl carbon of carboxymethyl groups (figure not shown). A substitution degree of 0.42 was determined by titration indicate a 63.6% of substitution of primary alcoholic groups.

Oxidation of the native polysaccharide with TEMPO-NaBr-NaOCl system

Reaction of the native polysaccharide from *Prosopis chilensis* with the TEMPO-NaBr-NaOCl gave a white solid in 95% yield readily soluble in water. Its FT-IR spectrum showed a signal at 1612 cm^{-1} assigned to the stretching vibration of carboxylate group and the ^{13}C NMR spectrum (Fig. not shown) of the derivative showed two signals at 175.3 and 175.7 ppm which were assigned to the carbonyl carbon of uronic acid residues. It is noteworthy that no signals due to carbonyl carbons of ketones were present at lower field. The uronic acid content (61.1%) determined by the carbazol method is very similar to the value expected (66.6%) for the total oxidation of free primary alcoholic groups. The ^{13}C NMR DEPT 135º spectrum (Fig. 3) showed only one inverted signal at 67.52 ppm which was assigned to C-6 of mannopyranosyl residues glycosylated at position *O*-6. The two signals at higher field in the DEPT spectrum of native galactomannan were missing.

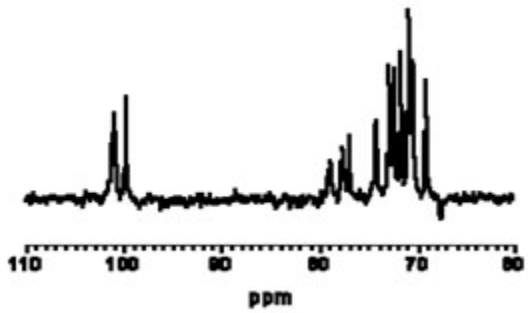

Fig. 3. ^{13}C NMR DEPT 135º spectrum of the oxidised galactomannan from *Prosopis chilensis* seeds.

Results indicated that the oxidation of free primary alcoholic group in the galactomannan was complete affording a polyuronic acid derivative (Fig. 4). According to Sierakowski et al.[27,28], TEMPO mediated oxidation of the galactomannans is more effective on mannopyrannosyl residues than on the galactopyranosyl residues. However, in the case of the galactomannan from *Cassia fastuosa* a degree of oxidation of 0.22 was obtained, significant amounts of mannose-mannose diads (galactose:mannose ratio 1.0:3.0) were present in the native polysaccharide, and the α-D-galactopyanosyl residues were distributed irregularly along the main chain. In the case of the regular galactomannan from *Prosopis chilensis* seeds, the side chain of galactopyranose was fully available for the oxidation.

In conclusion, TEMPO mediated oxidation of the slightly soluble galactomannan from seeds of *Prosopis chilrnsis* is a very effective chemical modification method to prepare ten times more aqueous soluble derivative with potential use in the food industry. This method is more selective than carboxymethylation for producing acidic derivatives.

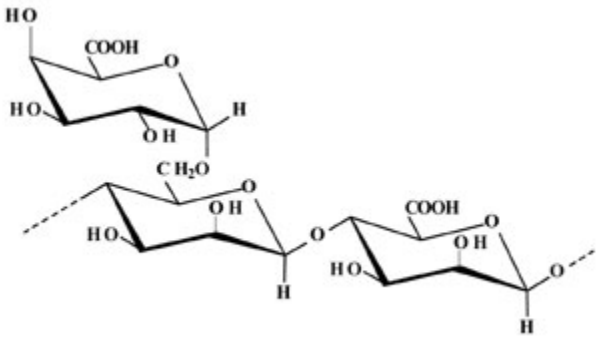

Fig. 4. Structure of the TEMPO oxidised polysaccharide from *Prosopis chilensis* seeds.

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