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SOME STRUCTURAL FEATURES OF A HETEROPOLYSACCHARIDE FROM THE LEAVES OF THE CACTUS *PERESKIA ACULEATA*

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Key Word Index—*Pereskia aculeata*: Cactacea; food additives; mucilage; acidic arabinorhamnogalactan; segmental motion.

Abstract—A homogenous water-soluble mucilaginous heteropolysaccharide containing 3.5% protein was isolated from the leaves of *Pereskia aculeata*. It contains arabinose, galactose, rhamnose and galacturonic acid in a molar ratio of 5.1:8.2:1.8:1.0 and, based on conventional polysaccharide analysis techniques, has a (1→4)-linked β-D-galactopyranosyl main chain partially substituted at O-3 by β-L-arabinopyranosyl units, which are, in turn, di-*O*-substituted at O-2 and O-4 by non-reducing end-groups of α-L-arabinofuranose. Also present are *O*-substituted units of galactopyranosyluronic acid, which are also present as non-reducing end-groups. They are then linked (1→2) to rhamnopyranosyl units. Aqueous solutions of the heteropolymer had a maximum viscosity at pH 4.5 and viscosity was reduced in the presence of salts over a wide range of pHs. The ^{13}C NMR spectrum of the polysaccharide in DMSO indicated a great difference between the elevated segmental motion of the arabinosyl side chains and that of the core, since signals of the former were sharp and those of the latter extremely broad.

INTRODUCTION

Pereskia aculeata Mill., known as 'ora pro nobis' in Brazil, is a native, bush-like cactus that reaches a height of 1 m, growing well in dry or wet tropical and subtropical regions. Its leaves are consumed by many people as a vegetable [1]. The protein content of the leaves was reported to be high by Almeida Filho and Cambraia [2] and the levels of the component essential amino acids, except for methionine, were found to be higher than the minima recommended by the FAO as necessary for human consumption [1].

A mucilaginous material, obtained by warm to hot aqueous extraction of the leaves, comprised 40% of the weight of acetone extracted powder. Such a substance has been isolated from many other plants and may act in the cell wall as a filler, as a carrier of lignin precursors, and may also serve to localize enzyme complexes [3].

The present study concerns the determination of the main chemical structures of the mucilaginous heteropolysaccharide of leaves of *P. aculeata*.

RESULTS AND DISCUSSION

Material extracted by hot water from the leaves was submitted to a Cetavlon precipitation procedure and then deproteinized. The product, P, was a glycoprotein containing only 3.5% of protein and on column chromatography on Sepharose 6B only one carbohydrate and protein-positive fraction was obtained. It was also homogeneous, as its Procion blue derivative, on cellulose acetate electrophoresis.

Aqueous solutions of P were found to be highly viscous. Its intrinsic viscosity was 7.0×10^2 ml/g at 20° and 7.9×10^2 ml/g at 25°. Maximum viscosity was observed at pH 4.5 and viscosity was less in the presence of salts over a wide pH range.

Component P contained arabinose, galactose, rhamnose and galacturonic acid in a mole ratio of 5.1:8.2:1.8:10 and controlled partial hydrolysis gave polymeric P-1 composed of units of arabinose, galactose, rhamnose and galacturonic acid in a mole ratio of 0.5:6.0:1.4:1. Most of the arabinosyl units were thus removed from the side chains leaving a core principally composed of galactosyl units.

Component P contained minor proportions of acetyl (6.7%), hexosamine (0.6%), phosphate (0.5%) and calcium (1.9%).

Preliminary information about the fine structure of component P was obtained by ^{13}C NMR spectroscopy. The spectrum obtained from a D_2O solution of P was ill-defined (Fig. 1A), but contained a low-field signal at δ 108.8 corresponding to C-1 of α-arabinofuranosyl units. However, using DMSO as solvent, several well-defined signals were obtained (Fig. 1B) whose shifts (δ 107.9, 83.7, 81.8, 76.9 and 61.2) corresponded to five carbons of α-arabinofuranosyl units [4]. These signals were superimposed on some very broad ones arising from the galactan core. Broad signals indicate that the molecular motion of the core is much less than that of the side chains, one related example being that of bovine nasal cartilage which contains proteoglycan and chondroitin sulphate, which give rise to ^{13}C NMR signals, and collagen, which does not [5]. Hitherto, although a difference has been observed in the segmental motion of the side chains and main chain of a branched-chain yeast mannan, this was not sufficient to give rise to differences in the respective line widths [6]. In the present spectrum (Fig. 1B), another sharp C-1 signal was also present at δ 101.5 and this could well have arisen from β-arabinopyranosyl units of the side chains (see later methylation evidence).

The ^{13}C NMR spectrum, in D_2O, of partially hydrolysed P1 (Fig. 1C) had a main C-1 signal at δ 105.9 whose relatively low field indicated that the galactopyranosyl

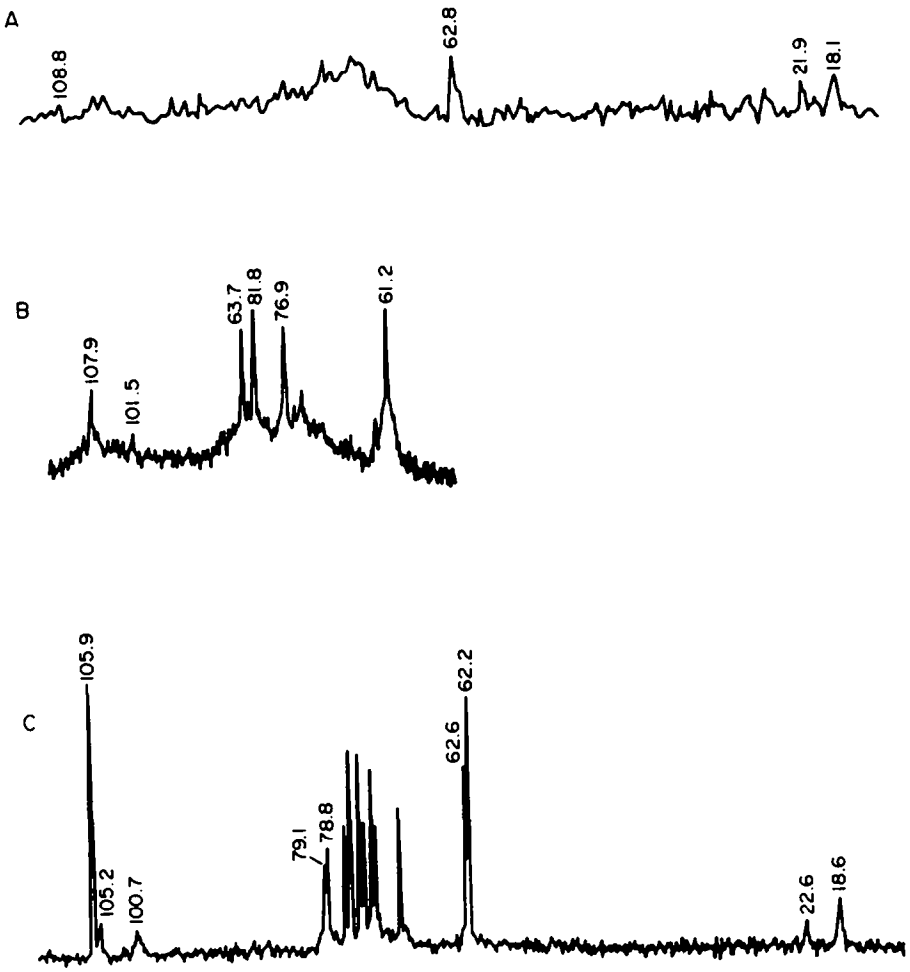


Fig. 1. ^{13}C NMR spectra of polysaccharides. Numerical values are in δ. A, P in deuterium oxide at 70°; B, P in DMSO at 33°; C, P-1 in deuterium oxide at 33°.

units had the β-form [7]. Since P-1 had a higher specific rotation (+ 49°) than its precursor P (− 29°), the contribution of the α-arabinofuranosyl units removed was negative thus indicating that they had the L-form.

Further studies on the detailed structure of Component P were carried by methylation analysis. The partially *O*-methylated alditol acetates were identified by GC/MS. As indicated in Table 1, the 2,3,5-tri-*O*-(20%) and 3-*O*-methyl (9.3%) derivatives of arabinitol were detected, showing that the majority of the L-arabinosyl residues were non-reducing end-groups with the furanosyl form. Araf residues must then serve as 2,5- and/or 2,4-di-*O*-substituents of the adjacent arabinosyl units. Such units could thus be responsible for the C-1 signal at δ 101.5 in spectrum 1B and could have either the β-arabinopyranosyl or β-arabinofuranosyl structure (see C-1 shifts of methyl β-arabinopyranoside and methyl β-arabinofuranoside, ref. [4]). The methylation data for analysis of P-1 favoured the presence of β-arabinopyranosyl units. P-1 contains arabinosyl residues not completely removed by partial hydrolysis and these gave rise to a trace of 2,3,4-tri-*O*-methylarabinitol acetate (Table 1) arising from pyranosyl non-reducing end-groups. These were likely exposed on partial hydrolysis by removal of arabinofuranosyl units from their O-2 and O-4 positions (the presence of 0.9% of 2-*O*-substituted arabinofuranosyl units in P was also indicated by the methylation data of Table 1).

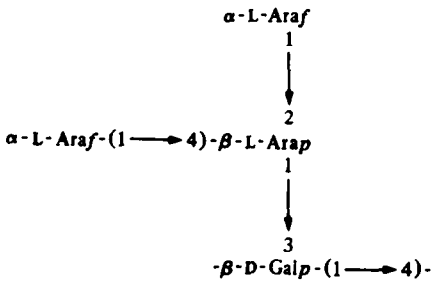
The structure of the main chain of Component P and the positions in which it is substituted by side chains was determined by comparison of methylation data obtained from it and its partially acid-degraded product P-1 (Table 1). The fragments obtained from P showed that galactopyranosyl units were present as non-reducing end-groups (2.3%) and as 4-*O*- (14.0%), 3-*O*- (3.7%) and 3,4-di-*O*-substituted (31.6%) structures. Removal of most of the arabinosyl residues gave P-1 which contained mainly non-reducing end-groups (10.1%) with 4-*O*-substituted residues (57.5%) of galactopyranose and thus contained the main chain of P with (1 → 4)-linked β-D-galactopyranosyl units. The decrease in the proportion of 3,4-di-*O*-substituted residues on going from P to P-1 and the above data on the structure of the arabinosyl side-chains showed that they were substitutents at O-3 of the main chain units of β-D-Galp-(1 → 4) as in structure **1**, a major component of P.

Since 2,3,4-tri-*O*-methylrhamnitol acetate (3.7%) was produced from P-1, it was clear that minor non-reducing

Table 1. Percentage composition of partially *O*-methylated alditol acetates formed on methylation of polysaccharides and oligosaccharides arising from *P. aculeata*

O-Methyl alditol acetate fragment	% Total*			
	Methylated P	Carboxymethyl-reduced, methylated P	Methylated P-1	Methylated, carboxymethyl-reduced, remethylated FA
2,3,4-Me_3-Ara	—	—	0.1	5.2
2,3,5-Me_3-Ara	20.0	21.0	—	13.0
3,5-Me_2-Ara	0.9	1.0	0.1	5.2
2,3-Me_2-Ara	0.6	0.5	—	9.0
3-Me-Ara	9.3	7.6	—	—
2,3,4-Me_3-Rha	—	—	3.7	3.9
3,4-Me_2-Rha	—	—	—	9.0
2-Me-Rha	1.9	2.8	—	—
3-Me-Rha	5.2	7.8	12.8	15.6
2,3,4,6-Me_4-Gal	2.3	4.0	10.1	11.7
2,3,6-Me_3-Gal	14.0	19.2	57.5	7.8
2,4,6-Me_3-Gal	3.7	0.3	—	—
2,6-Me_2-Gal	31.6	25.6	2.4	7.8
3,6-Me_2-Gal	—	—	0.4	5.2
2,3-Me_2-Gal	—	0.8	0.9	6.5
2,4-Me_2-Gal	—	0.4	—	—
2-Me-Gal	—	1.2	—	—
2,3,6-Me_3-Glu	3.7	0.1	0.3	—

*In terms of peak areas obtained on GC/MS.



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end-groups of rhamnopyranose were uncovered by partial hydrolysis by removal from 3,4-di-*O*-substituted rhamnopyranosyl units of residues of arabinose and/or rhamnose. In view of the increase from 5.2 to 12.8% of the 3-*O*-methyl rhamnitol derivative on going from P to P-1, the 2,4-di-*O*-substituted rhamnopyranosyl units were relatively acid stable and located in the galactopyranose-containing core.

Component P was partially hydrolysed with acid under conditions stronger than those used for formation of P-1 and the mixture of oligosaccharides containing uronic acid was isolated via chromatography on a column of anionic exchange resin. The mixture was successively methylated, carboxymethyl reduced, remethylated and converted to *O*-methylalditol acetates, which were examined by GC/MS (Table 1). Although 3,4-di-*O*-methylrhamnitol acetate was not a product in the methylation of P, it constituted 9.0% of the product arising from the acidic oligosaccharides (FA). Thus galactopyranosyl (uronic acid) units were linked (1→2) to those of rhamnopyranose in Component P. It is noteworthy that the ^{13}C NMR spectrum of P-1 contains a C-1 signal at δ 100.7, close to that at δ 100.6 of a (1→4)-linked α-D-galactopyranosyl (uronic acid) polymer [8].

The nature of the galactopyranosyl (uronic acid) units in P was determined by comparison of *O*-methylgalactitol acetates, formed from methylated P, with those arising from methylated P, that were reduced with lithium aluminium hydride to convert CO_2Me to CH_2OH groups. It can be seen (Table 1) that reduction gave rise to an increased proportion of 2,3,4,6-tetra-*O*-methyl derivative and the appearance of 2-*O*-, 2,3- and 2,4-di-*O*-methyl derivatives, which correspond respectively to the residues of galactopyranosyl (uronic acid), 3,4-di-*O*-, 4-*O*- and 3-*O*-substituted, that are nonreducing ends.

O-Acetyl groups are minor components in Component P and the partial acid hydrolysis product P-1, as their ^{13}C NMR spectra contain typical Me signals at δ 21.9–22.6 (Fig. 1A and C), which are removed by alkaline hydrolysis. It is hoped to determine their exact locations in the molecule in a future study.

EXPERIMENTAL

General procedures. Evaporation of solns was carried out at 35–40° under reduced pressure. Protein was estimated using the micro-Kjeldahl nitrogen assay [9], total carbohydrate by the phenol–sulphuric acid method [10], uronic acid by the carbazole

method [11], *O*-acetyl by the procedure of Hestrin [12], phosphate according to Bartlett [13], calcium by the method of Clark and Collip [14] and hexosamine by that of Dische [15]. Galactose in the hydrolysate of P was shown to have the D-form by the method of Amaral *et al.* [16].

Isolation of Component P. Leaves of *P. aculeata* (271 *g*) were extracted in a Soxhlet successively with EtOH and Me_2CO, then dried and pulverized in a Wiley mill (60 mesh), and completely defatted with refluxing C_6H_6–EtOH (2:1). The material (30 g) was stirred in H_2O (600 ml) at 70° for 12 hr, this extraction being repeated twice and the combined filtered extracts dialysed against distilled water. Addition of EtOH (4 vol.) precipitated polysaccharide which was isolated via successive centrifugation, and washes with EtOH and Me_2CO. The dried product (5.9 g), called Component P, was dissolved in H_2O (700 ml) which was added to an equal vol. of 0.1 M sodium tetraborate made up to 0.15 M with Cetavlon. The pH was then adjusted from 8.0 to 9.0 with aq. NaOH and the precipitate which formed was isolated, dissolved in 2 M HOAc, added to 3 vol. of EtOH and the precipitated polysaccharide isolated (yield 5.9 g). Due to the quantitative yield on Cetavlon precipitation and since no protein was removed using the Sevag procedure [17], Component P was pure following aq. extraction followed by EtOH precipitation.

Homogeneity of Component P by gel filtration and by electrophoresis. Component P (45 mg) in 25 mM Tris–HCl was applied to a column (40 × 2.0 cm) of Sepharose 6B which was eluted with the same buffer. Fractions of 2.5 ml were collected and assayed for carbohydrate and protein.

Component P was dyed with Procion blue and electrophoresis carried out on cellulose acetate according to the method of Dudman and Bishop [18].

Partial hydrolysis of P to form P-1. Component P (235 mg) in 0.02 M trifluoroacetic acid (10 ml) was maintained at 100° for 2 hr and then added to 4 vol. of EtOH. The precipitated polysaccharide, P-1, was centrifuged and washed successively with EtOH and Me_2CO. Yield 115 mg.

Partial hydrolysis of P to yield acidic oligosaccharides. Component P (50 mg) in M trifluoroacetic acid (10 ml) was heated at 98° for 5 hr. Following evaporation, the hydrolysate was applied to a column (15 × 2.0 cm) of AG-X10 (OAc^- form; 200/400 mesh) and the neutral sugars (30 mg) removed by elution with H_2O. The acidic material (FA; 13 mg) was eluted with 30% aq. HOAc.

Viscosity determination of Component P. The viscosities of P in soln were determined using an Ostwald-type viscometer at aq. concentrations of 0.215–1.28% at 20° and 25°. A plot of reduced viscosity against polysaccharide concentration was used to determine the intrinsic viscosity [7, 19]. The relative and reduced viscosity were determined in the presence of NaCl, NaBr, NaI, KCl, KBr, KI (1.0–3.0 M) and at pHs of 1.5–12.5 [20].

Determination of monosaccharide composition. Neutral sugar and uronic acid components were detected in polysaccharide hydrolysates (M trifluoroacetic acid, 100°, 5 hr) by PC using appropriate standards with solvent: C_6H_6–*n*-BuOH–C_5H_5N–H_2O (1:5:3:3) and spray: alkaline $AgNO_3$ [21]. Quantitation was achieved by conversion of the hydrolysate, by successive $NaBH_4$ reduction and acetylation in hot C_5H_5N–Ac_2O, to a mixture of alditol acetates. This was examined using a conventional GC column of 3% ECNSS-M on Gaschrom Q (0.4 i.d × 120 cm) at 180° [22]. Amino acids formed on hydrolysis (6 M HCl, 105°, 18 hr) were examined by TLC on silica gel G-60 with solvent: PhOH–H_2O (3:1) and spray: ninhydrin in Me_2CO [23].

Methylation analysis of oligosaccharides and polysaccharides. Polysaccharides P and P-1 and the oligosaccharide mixture FA were fully methylated by successive treatments by the Haworth [24] and Kuhn [25] procedures.

In the case of P and FA, the per-*O*-methylated products were treated with lithium hydride in tetrahydrofuran to reduce carboxymethyl to hydroxymethyl groups [26]. The product from FA was remethylated by the Kuhn procedure.

O-Methylated products were hydrolysed in 72% (v/v) aq. H_2SO_4 at 25° for 1 hr, the hydrolysate then diluted with H_2O to form an 8% soln of H_2SO_4, which was heated at 100° for 4 hr [27]. The resulting mixture of partially *O*-methylated aldoses was converted to one of partially *O*-methylated alditol acetates, as described above for aldoses, and analysed by GC/MS, peaks being identified by their electron impact EIMS patterns and typical retention times [28]. GC/MS was performed using a Finnegan 4000 unit linked to an Incos 2300 Data System, incorporating a capillary column of 1:3 OV-17 blended with OV-225 [29] (0.25 mm i.d × 30 m). The temperature was programmed rapidly from 50° (at 40°/min) to 182° (then hold). Scans were made for mass 40–420 every 2 sec at 70 eV.

^{13}C NMR spectroscopy. The spectrum of component P was recorded with a Varian XL-100-15 spectrometer in the Fourier-transform mode for a soln of the sample (100 mg) in D_2O (0.85 ml) contained in a coaxial tube fitting snugly within a tube (0.12 diam. × 20.3 cm) maintained at 70°. The spectral width (s.w.) was 5000 Hz, the acquisition time (a.q.) 0.4 sec, the pulse width (p.w.) 9.5 μsec, and the number of transients (n.s.) 69 080. Chemical shifts are expressed relative to the resonance of Me_4Si, obtained in a separate experiment. Spectra of P in DMSO (60 mg) and Fraction P-1 (18 mg) in D_2O at 33° were obtained using a Bruker AM-360-WB NMR spectrometer. Solns (2 ml) were contained in tubes of 0.10 cm diam. Spectral parameters were n.s. 88 496 and 79 488, respectively, s.w. 18 519 Hz, a.q. 0.44 sec and p.w. 21.0 μsec. Chemical shifts were corrected to those obtained at 70° using the 100 MHz machine.

Specific rotations. These were measured at 25° at a polysaccharide concentration of 0.15% in water.

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