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Structural studies of acidic xylans exuded from leaves of the monocotyledonous plants *Phormium tenax and Phormium cookianum*

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Abstract

The polysaccharides exuded from both *Phormium tenax* and *Phormium cookianum* leaves are acidic xylans with molecular weights in the range 1.3×10^6–1.7×10^6 Da. ^{1}H and ^{13}C NMR spectra show that in both cases the backbone comprises 1,4-linked β-D-Xyl*p* units and their substituted products, including a structural unit that is both 2-*O*-substituted and 3-*O*-acetylated. The sidechains contain α-D-GlcA and are terminated by β-D-Xyl*p* and α-L-Ara*f* units in relative proportions that differ between plant varieties. The polysaccharides are so highly branched that chain-terminating units account for approximately half of all structural units. These properties place the *Phormium* polysaccharides in a class which includes brea, sapote and yabo gums.

Keywords: *Phormium*; Xylan; Mucilage; ^{13}C NMR; ^{1}H NMR

1. Introduction

Phormium is a genus of monocotyledonous plants within the family Phormiaceae J.G. Agardh, of the order Asparagales (Watson & Dallwitz, 1992 onwards). The Maori people of New Zealand collected mucilage exuded from the base of *Phormium* leaves and applied it to burns and wounds (Goldie, 1904). The mucilage, known to Maori as 'pia harakeke', can be dried to a gum which presents many features in common with gum arabic (Aitken, 1927). Interest in pia harakeke has been revived recently, when cosmetics manufacturers began using it to thicken skin-care products.

Pia harakeke is an acidic xylan. McIlroy (1951) reported a composition of 45% D-xylose (Xyl), 42% D-glucuronic acid (GlcA), 4% lignin and 5% ash. Arabinose (Ara) was not detected in McIlroy's analysis, but has been found in all of the other xylan exudates for which data are available (Table 1). These results suggest that arabinose may occur in pia harakeke also, providing additional incentive to reinvestigate its chemical composition.

All known xylan exudates have highly branched structures, unlike the acidic xylans found in angiosperm wood (Evtuguin, Tomás, Silva, & Neto, 2003; Teleman, Tenkanen, Jacobs, & Dahlman, 2002), legume husks (Swamy & Salimath, 1990) and wheat straw (Sun, Lawther, & Banks, 1996). Intermediate degrees of branching have been found in cereal arabinoxylans (Annison, Choct, & Cheetham, 1992; Bengtsson, Åman, & Andersson, 1992). We used NMR spectroscopy to characterise the degree of branching in pia harakeke, for comparison with other xylans.

Botanists recognise just two species of *Phormium*: *Phormium tenax* J. R. et G. Forst. (New Zealand flax) and *Phormium cookianum* Le Jolis (mountain flax). However, there is considerable variation in form within each species, and the species hybridise. We chose the traditional varieties Awahou, Kohunga and Tapoto as examples of *P. tenax* and Whaririki as an example of *P. cookianum*.

2. Materials and methods

2.1. Isolation of polysaccharides

Mucilage was collected in February 2000 from plants growing in the National New Zealand Flax Collection at Landcare Research, Lincoln, New Zealand (Scheele & Walls, 1994). One ramet of each variety was cut at approximately

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Table 1
Sources of acidic xylan exudates

Plant species	Name of gum	Uronic acids (% dry weight)	Reference
Monocotyledons:			
Livistona chinensis	–[a]	12	Maurer-Menestrina et al. (2003)
Puya chilensis	Chagual	16[b]	Hamilton et al. (1957)
Watsonia versveldii	–[a]	29	Shaw & Stephen (1966)
Dicotyledons:			
Cercidium australe	Brea	24	Cerezo et al. (1969)
Cercidium praecox	Yabo	25	León de Pinto et al. (1994)
Sapota achras	Sapote	27	Dutton & Kabir (1973)

[a] No common name.
[b] Reported as 27% of a biose GlcA–Xyl.

5 cm above ground level. The lower, fused section of each leaf was split open and mucilage was scraped from the surface of the leaf, dissolved in water at 80 °C, dialysed (molecular weight cut off 12–14,000) exhaustively against distilled water and freeze-dried.

2.2. Molecular weights

Molecular weight distributions were determined by size-exclusion chromatography coupled with multi-angle laser light scattering (SEC-MALLS). The SEC-MALLS system consisted of a Waters 2690 Alliance separations module, a DAWN-EOS multi-angle laser light scattering detector with a laser at 690 nm (Wyatt Technology Corp., Santa Barbara, CA) and a Waters 2410 refractive index monitor. Samples (2 mg mL^{-1} in eluant) were filtered (0.45 μm) before injection (50 μL) and eluted with 0.1 M $LiNO_3$ containing 0.02% NaN_3 (0.7 mL/min) from two columns (Shodex OHpak SB805 HQ and OHpak SB804 HQ, 300×7.8 mm, Showa Denko, Miniato, Japan) connected in series. Data for molecular weight determination and conformation were analyzed using ASTRA software (Version 4.783.04, Wyatt Technology Corp.) using a d*n*/d*c* value of 0.145 mL/g which was determined experimentally. Weight recoveries from the column as judged by refractive index were >90%. Entries in Table 2 are averages of duplicate determinations.

Table 2
Yield and composition of the polysaccharides obtained from four cultivars

Cultivar	Yield of gum (mg g^{-1})[a]	Molar mass (10^6 Da)	Total sugar (% of dry wt)	Uronic acid[b] (% of dry wt)
Awahou	1.45	1.32	88.3	32.2
Kohunga	2.56	1.28	89.0	30.9
Tapoto	2.43	1.58	86.7	30.5
Whahariki	2.15	1.68	88.3	33.3

[a] Dry weight of gum divided by fresh weight of leaf.
[b] Determined colorimetrically.

2.3. Chemical analyses

The total sugar content was determined using the phenol–sulphuric acid method with a 2:1 w/w mixture of Xyl and GlcA as a standard (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956). The uronic acid content was determined by the 3-phenylphenol method using GlcA as the standard (Blumenkrantz & Asboe-Hanson, 1973). Entries in Table 2 for total sugar and uronic acid contents are averages of duplicate determinations.

Constituent sugar compositions were determined by gas chromatography–mass spectrometry (GC–MS) following methanolysis and conversion of the methyl glycosides to their corresponding trimethylsilyl derivatives, essentially as described by McConville & Bacic (1989). Briefly, freeze-dried mucilage was dissolved in distilled water at 1 mg mL^{-1} and aliquots containing ~50 μg material were dispensed into vials and freeze-dried for analysis. Samples were dissolved in 1 M methanolic HCl (400 μL) and methyl acetate (100 μL) and heated in sealed tubes (80 °C, 16 h). After evaporation, the samples were dissolved in methanol (250 μL), pyridine (25 μL) and acetic anhydride (25 μL) and incubated at 20 °C for 15 min to re-*N*-acetylate any potential *N*-acetylated sugars (none were detected). The resulting methyl glycosides were converted to their trimemethylsilyl derivatives with Tri-Sil reagent (100 μL, Pierce, IL, USA) at 70 °C for 10 min. The derivatives were separated by GC on an HP-Ultra2 capillary column (30 m×0.25 mm i.d., 0.25 μm film thickness) with the GC oven programmed from 50 °C (held for 1 min) to 140 °C at a rate of 25 °C min^{-1}, and then to 250 °C at a rate of 3 °C min^{-1}, and analysed by MS using a Hewlett Packard 5970 MSD. Identifications were based on peak retention times and electron impact mass spectra compared with trimethylsilyl derivatives of standard sugars. Quantifications were made from peak areas using response factors calculated from trimethylsilyl derivatives of standard sugars.

2.4. NMR spectroscopy

The ^1H and ^{13}C NMR spectra were acquired at 500 and 125.7 MHz, respectively, using a Varian Unity-500 spectrometer. Samples were dissolved in 99.6 at.% D_2O, at concentrations of 5 mg in 0.5 mL in a 5 mm o.d. tube for ^1H NMR and 100 mg in 3 mL in a 10 mm o.d. tube for ^{13}C NMR. The ^1H NMR spectra were acquired at 70 °C with each 90° pulse followed by 4.1 s data acquisition and a 6 s recovery delay. Signals were averaged over 7 min. The ^{13}C NMR spectra were acquired at 80 °C with each 90° pulse followed by a 1.1 s data acquisition time and a 0.3 s recovery delay. Signals were averaged over at least 3 h. Chemical shifts were measured relative to an internal standard of Me_2SO 39.5 ppm (^{13}C NMR) or relative to the acetyl signal of the polysaccharide at 2.17 ppm (^1H NMR).

HH-COSY 2D plots were acquired but not shown because they were too crowded and poorly resolved to be of general use in assigning ^1H NMR signals. The 2D plots were used for two specific purposes described in Section 3.3.

Table 3
Constituent sugar composition of the polysaccharides obtained from four cultivars

Cultivar	Sugar composition (wt%)		
	Ara	Xyl	GlcA
Awahou	13.1	77.4	9.5
Kohunga	7.8	81.4	10.8
Tapoto	4.9	87.2	7.9
Whahariki	26.7	64.1	9.2

3. Results and discussion

3.1. Yields and composition

Yields (Table 2) corresponded to between 1.0 and 1.6 g dry gum obtained from each ramet. Weight-averaged molecular weights (Table 2) were all greater than 10^6 Da. Each polysaccharide eluted as a single peak on SEC-MALLS. Total sugar contents (Table 2) were similar to those reported by McIlroy (1951). The polysaccharides were not converted to acidic forms, so cations accounted for at least some of the remainder of the dry weight. The wt% uronic acid contents reported in Table 2, in the range 30.5–33.3% of dry weight, were lower than the value (42 wt% of dry weight) reported by McIlroy (1951) and constituent sugar data (Table 3) gave values which were lower again (7.9–10.8%). One possible explanation for the much lower uronic acid content estimated by the constituent sugar analysis is incomplete derivatisation of uronic acid residues during methanolysis, due to the resistance of GlcA–Xyl residues to hydrolysis. However, the estimates of uronic acid content by ^{1}H NMR spectroscopy (see Section 3.2) were closer to those from constituent sugar analysis and suggested that the colorimetric assay overestimated the uronic acid content.

Constituent sugar analysis of samples gave only Ara, Xyl and GlcA (Table 3). The Ara:Xyl ratio varied widely, from 6:100 to 42:100, so it is conceivable that McIlroy (1951) chose a *P. tenax* plant in which the arabinose content was below the detection limit. No 4-*O*-methyl-GlcA was detected in our constituent sugar analyses, although it accounts for all of the uronic acids in sapote gum (Dutton & Kabir, 1973), and approximately one third of the uronic acids in brea gum (Cerezo, Stacey & Webber, 1969) and *Livistona* exudate (Maurer-Menestrina, Sassaki, Simas, Gorin, & Iacomini, 2003).

3.2. ^{13}C NMR spectroscopy

The tallest peaks in the spectrum of the Tapoto polysaccharide (Fig. 1(a)) were assigned to xylopyranose residues in terminal positions, i.e. β-D-Xyl*p*-(1→, abbreviated to X*t* in the labels used in Fig. 1. The chemical shifts (Table 4) were similar to those reported by Kovac, Hirsch, Shashkov, Usov, and Yarotsky (1980) for xylo-oligosaccharides. The same peaks appeared in the spectrum of the Whahariki polysaccharide (Fig. 1(b)), along with peaks of similar height assigned to arabinofuranose residues in terminal positions, i.e. α-L-Ara*f*-(1→, abbreviated to A*t* in the labels used in Fig. 1(b). The chemical shifts (Table 4) were similar to those reported by Hoffman, Roza, Maat, Kamerling, & Vliegenthart (1991); Ebringerová, Hromádková, Alföldi and Berth (1992). Peaks assigned to C-1 of both β-D-Xyl*p*-(1→ and α-L-Ara*f*-(1→ appeared in clusters of three, suggesting three distinct points of attachment. Spectra of the Awahou and Kohunga polysaccharides (not shown) contained peaks assigned to the same terminal units, in different relative proportions.

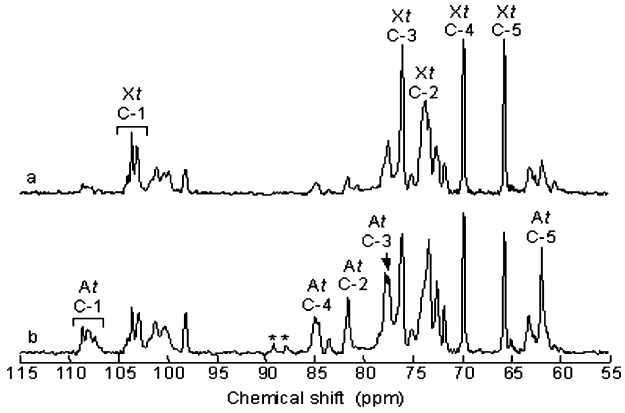

Fig. 1. ^{13}C NMR spectra of polysaccharides from *Phormium* varieties: (a) Tapoto, (b) Whahariki. Labels A*t* and X*t* refer to arabinose and xylose chain-terminating units, respectively. Stars mark signals assigned to C-2 of →2)-α-L-Ara*f*-(1→ units. Signal assignments for other units are discussed in Section 3.2.

A peak at 98.2 ppm was assigned to C-1 of α-D-GlcA as in ^{13}C NMR spectra of yabo gum (León de Pinto, Martínez & Rivas, 1994). However, Dutton & Kabir (1973) found C-2 substitution of 40% of the GlcA structural units in sapote gum, and this might affect the chemical shift of C-1, so it is possible that GlcA might contribute additional peaks in the vicinity. Those peaks might overlap signals in the range 100–102 ppm, assigned to both xylan chain units →4)-β-D-Xyl*p*-(1→ and xylan branching units (León de Pinto et al., 1994), so it is not possible to assign GlcA peaks in this region or to use C-1 peak areas to estimate the uronic acid content. Instead, we divided the C-1 region into three chemical-shift ranges assigned α-L-Ara*f*-(1→ (105 to 110 ppm), β-D-Xyl*p*-(1→ (102.5–105 ppm) and all other units (97–102.5 ppm). We then used signal areas to estimate the mol% structural units for each of the four varieties of *Phormium* (Fig. 2). Best-fit lines were drawn through the data points, and the slopes suggested that increasing the arabinose content caused replacement of some of the β-D-Xyl*p*-(1→ units by α-L-Ara*f*-(1→ units, with little

Table 4
^{13}C NMR chemical shifts

Unit	C-1	C-2	C-3	C-4	C-5
*Xyl*p-*(1→*	103.1 103.6 104.1	73.7	76.1	69.8	65.7
Araf-(1→	107.3 108.1 108.6	81.6	77.4	84.9	61.9

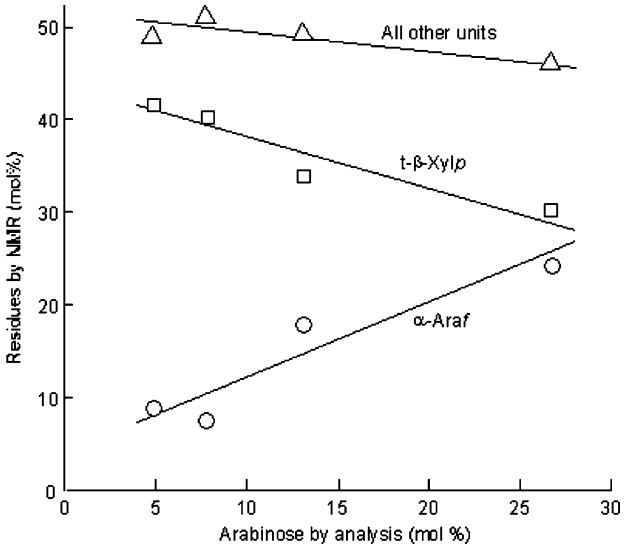

Fig. 2. Compositions of pia harakeke, determined by ^{13}C NMR spectroscopy, plotted against compositions determined by constituent sugar analysis. Data points are shown, from left to right, for Tapoto, Kohunga, Awahou and Whahariki. The lines represent linear least-squares fits.

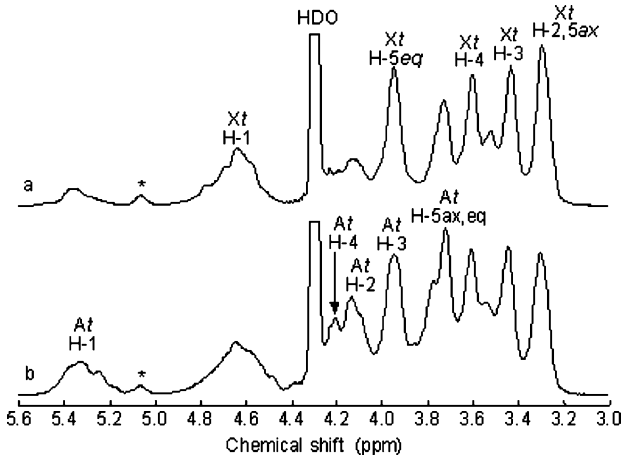

Fig. 3. Proton NMR spectra of polysaccharides from *Phormium* varieties: (a) Tapoto, (b) Whahariki. Labels A*t* and X*t* refer to arabinose and xylose chain-terminating units, respectively. Stars mark a signal assigned to H-3 of →4)-β-D-Xyl*p*-(1→ units substituted by GlcA on *O*−2 and acetyl on *O*-3. Signal assignments for other units are discussed in Section 3.3.

or no effect on the other structural units. The arabinose content of the pia harakeke might be a useful chemotaxonomic marker for classification of *Phormium* varieties.

Peaks outside the C-1 region provided further information about the chemical structure. Peaks at 87.9 and 89.2 ppm in the spectrum of the Whahariki polysaccharide (Fig. 1(b)) were both assigned to C-2 of →2)-α-L-Ara*f*-(1→ units in various sidechains (Ebringerová et al., 1992). The areas of those peaks suggested that →2)-α-L-Araf-(1→ units accounted for approximately 20% of all arabinose structural units. We attributed the lack of such signals in Fig. 1(a) to the much lower arabinose content in the Tapoto polysaccharide. A peak at 77.5 ppm in Fig. 1(a) was assigned to C-4 of →4)-β-D-Xyl*p*-(1→ chain units and also to the corresponding *O*−2- or *O*-3-substituted branching units (Hoffman et al., 1991; Kovac et al., 1980; León de Pinto et al., 1994; Vignon & Gey, 1998). The absence of a C-3 peak at 88.9 ppm precluded →3)-β-D-Xyl*p*-(1→ chain units (Yamagaki, Maeda, Kanazawa, Ishizuka, & Nakanishi, 1997). A small peak at 60.5 ppm was tentatively assigned to methoxyl carbon in 4-Me-*O*-α-D-GlcA units (León de Pinto et al., 1994), although the constituent sugar analysis did not detect such units (Section 3.1). A peak at 21.4 ppm, outside the range illustrated in Fig. 1, was assigned to acetyl groups. Teleman, Lundqvist, Tjerneld, Stålbrand, and Dahlman (2000) reported the same chemical shift for acetyl groups in the 3-*O*-acetylated xylose structural units of aspen xylan. Many other peaks remained unassigned because the chemical shifts were non-specific.

3.3. Proton NMR spectroscopy

The tallest peaks in the proton NMR spectrum of the Tapoto polysaccharide (Fig. 3(a)) were assigned to β-D-Xyl*p*-(1→ and labeled X*t*. The same peaks appeared in the spectrum of the Whahariki polysaccharide, along with peaks assigned to α-L-Ara*f*-(1→ and labeled A*t*. Peaks assigned to H-2 and H-5*ax* of X*t* were incompletely resolved in 1D spectra (Fig. 3), but HH-COSY 2D NMR plots (not shown) included cross-peaks that provided more precise chemical shifts. All chemical shifts for X*t* and A*t* (Table 5) were consistent with values published by Kormalink, Hoffmann, Gruppen, Voragen, Kamerling and Vliegenthart (1993); Teleman et al. (2000) for oligosaccharides.

A peak at 5.07 ppm was assigned to H-3 of →4)-β-D-Xyl*p*-(1→ chain units substituted by GlcA on *O*−2 and acetyl on *O*-3 (Evtuguin et al., 2003; Teleman et al., 2000;). An HH-COSY plot for the Tapoto polysaccharide was used to locate H-1 (4.73-4.83 ppm), H-2 (3.76 ppm) and H-4 (3.95 ppm) in the same structural unit. These chemical shifts were all consistent with those published by Teleman et al. (2000); Evtuguin et al. (2003). A peak at 2.17 ppm (not shown in Fig. 3) confirmed the presence of acetyl groups. The area of the peak at 5.07 ppm indicated acetylated branching units at a level of approximately 5 mol% in all four polysaccharides. Evtuguin et al. (2003) reported a chemical shift of 3.70 ppm for H-3 in the corresponding acetyl-free branching unit. Unfortunately, this region was too crowded, even in the HH-COSY plot, for positive identification of such a peak.

A cluster of peaks between 5.24 and 5.42 ppm in the proton NMR spectrum of the Tapoto polysaccharide (Fig. 3(a)) was assigned to H-1 of α-D-GlcA, with peaks at the top end of this range indicating *O*−2-substitution (Evtuguin et al., 2003). A similar cluster appeared in each of the other spectra, but was

Table 5
Proton NMR chemical shifts

Unit	H-1	H-2	H-3	H-4	H-5ax	H-5eq
Xylp-(1→	4.56–4.74	3.33	3.46	3.63	3.97	3.30
Araf-(1→	5.26–5.32	4.16	3.95	4.22	3.79	3.73

enhanced by larger contributions from H-1 of α-L-Ara*f*-(1→. The peak area was plotted against the arabinose content, and extrapolated back to 13 mol% of GlcA units for a hypothetical arabinose-free polysaccharide. This estimate of the uronic acid content was a little higher than values indicated by constituent sugar analyses (Table 3). Vignon & Gey (1998) observed discrepancies of similar magnitude in a study of jute xylan, and attributed them to incomplete hydrolysis of GlcA units under the conditions of constituent sugar analysis.

3.4. A structural model

The results reported above point to a highly branched structure, with a mixture of β-D-Xyl*p* and α-L-Ara*f* chain-terminating units accounting for approximately half of all structural units in pia harakeke from all four varieties of *Phormium* (Fig. 2). The core of the polysaccharide is constructed from →4)-β-D-Xyl*p*-(1→ chain units, some of which are also substituted at *O*-2- and/or *O*−3, with branching units. The branching units include α-*O*-GlcA-(1→2)-Xyl as found in other xylan exudates, i.e., chagual (Hamilton, Spriestersbach & Smith, 1957), brea (Cerezo et al., 1969), yabo (León de Pinto et al., 1994) and *Livistona* exudate (Maurer-Menestrina et al., 2003). At least some of the α-D-GlcA units are substituted at *O*-2, possibly by β-D-Xyl*p* or α-L-Ara*f* as in brea (Cerezo et al., 1969). Our NMR results, supported by the constituent sugar analyses, suggest that uronic acids account for 13% of sugar residues. This value is too low for GlcA to be contained in every sidechain. We conclude that pia harakeke contains branches in which xylose in a sidechain is linked to xylose in the backbone.

The composition of pia harakeke is similar to those of brea, sapote and yabo gums (Cerezo et al., 1969; Dutton & Kabir, 1973; León de Pinto et al., 1994) but it lacks the fucose and rhamnose found in *Livistona* exudate (Maurer-Menestrina et al., 2003), and it also lacks the galactose found in chagual gum (Hamilton et al., 1957) and *Watsonia* exudate (Shaw & Stephen, 1966). A highly-branched acidic galactoxylan has been extracted from linseed (Muraliksrishna, Salimath, & Tharanathan, 1987), but pia harakeke lacks the galactose found in that polysaccharide.

4. Conclusions

The polysaccharides exuded from *Phormium* leaves show structural features in common with highly-branched xylan exudates from other species. Samples from different *Phormium* varieties show differences in the relative proportions of arabinose and xylose, but those differences are superficial in that they are mostly confined to the terminating units of sidebranches.

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