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The Yariv reagent: Behaviour in different solvents and interaction with a gum arabic arabinogalactanprotein

B.S. Paulsen[a,*], D.J. Craik[b], D.E. Dunstan[c], B.A. Stone[d,1], A. Bacic[e]

[a] Department of Pharmacy, Section Pharmacognosy, University of Oslo, 0316 Oslo, Norway
[b] Institute for Molecular Bioscience, The University of Queensland, Brisbane, 4072 Queensland, Australia
[c] Department of Chemical and Biomolecular Engineering, University of Melbourne, Parkville, 3010 Victoria, Australia
[d] School of Biochemistry, La Trobe University, Bundoora, Melbourne, 3083 Victoria, Australia
[e] ARC Centre of Excellence in Plant Cell Walls, Plant Cell Biology Research Centre, School of Botany, University of Melbourne, Parkville, 3010 Victoria, Australia

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ABSTRACT

The β-D-Glc Yariv reagent is frequently used to isolate and to study the structure of arabinogalactan-proteins with the arabinogalactan type II structure. The present paper describes the aggregation features of the Yariv reagent in water, salt solutions and in organic solvents as determined by NMR, absorption spectroscopy and light scattering experiments. The results indicate that in water the Yariv reagent forms aggregates of up to 300 units and in 1% aqueous NaCl the degree of aggregation is approx. 150. The aggregates are formed both by H-bonds and hydrophobic interactions, the former appearing to be of most importance in water.

The interaction between the Yariv reagent and an AGP fraction from gum arabic, showed a degree of aggregation of the Yariv reagent when using 1% NaCl to be of approx. 150 units, whereas disruption of the aggregate took place in 10% NaCl with an aggregation number of approx. 100.

Partial acid hydrolysis of an AGP from gum Arabic (*Acacia Senegal*) and analyses of the linkage types remaining indicated that a certain length of (1 → 3)-β-linked galactose units was necessary for binding between the Yariv reagent and the AGP. This is in accordance to what also was recently observed by Kitazawa et al. (2013).

1. Introduction

Yariv, Rapport, and Graf (1962) prepared a series of artificial antigens for detecting antibodies to carbohydrates by coupling diazotised 4-amino-phenyl glycosides with phloroglucinol (Table 1). Later, Yariv, Lis, and Katchalski (1967) and Jermyn and Yeow (1975) observed that these reagents precipitated arabinogalactan-proteins (AGPs) in a wide range of plant extracts. Subsequently the β-glucosyl Yariv reagent (β-D-Glc Yariv reagent) has been widely used in the purification of AGPs (for example, see Gane et al., 1995), and through its strong absorption in the visible spectrum (λ_{max} 430 nm) in their quantification (Van Holst & Clarke, 1985), detection in gels (Van Holst & Clarke, 1986) and in plant sections by light microscopy (Schopfer, 1990). Notwithstanding the utility of the β-D-Glc-Yariv reagent in these applications, the molecular basis for its specific interaction with certain AGPs is not fully understood.

The interaction depends both on the state of the Yariv reagent in solution and the chemical structure and organisation of the AGP molecule. The reaction is usually performed in a 1% aqueous solution and the precipitated complex can be dissociated by adding either dimethylformamide (DMF) or by adding NaCl solution to 10% (w/v). Complex formation occurs only with β-D or α-L-linked glycopyranosyl Yariv reagents, so that the saccharide moiety is implicated in the binding reaction. In addition, the OH group at C(O)2 must be in the D-gluco configuration and the diazo-group which substitutes the phenylglycoside must be at the C4 position of the phenyl ring (Jermyn, 1978a; Jermyn & Yeow, 1975).

In aqueous solution the β-Glc Yariv reagent is proposed to be highly aggregated (5–50 monomers per aggregate) either by stacking or overlapping of the planar benzene rings (Nothnagel & Lyon, 1986; Woods, Lilley, & Jermyn, 1978). High temperatures and solvents which disrupt either hydrogen bonding, dipolar or hydrophobic interactions, such as 10% NaCl, 6 M guanidine HCl, 6 M urea or 1% SDS, cause Yariv reagent aggregates to dissociate and furthermore, prevent them from binding to AGPs (Woods et al., 1978).

* Corresponding author. Tel.: +47 22 85 65 72.
E-mail address: b.s.paulsen@farmasi.uio.no (B.S. Paulsen).

[1] Subsequent to the completion of this work Professor Bruce A. Stone passed away (28 June 2008) so we dedicate this work to his memory since AGPs were one of his favourite molecules.

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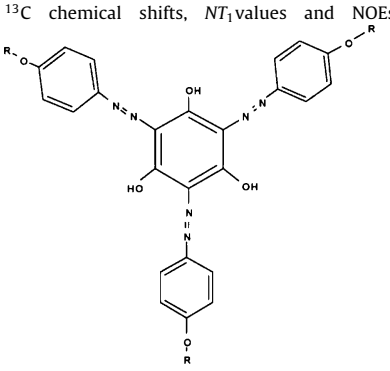
Table 1
^{13}C chemical shifts, NT_1 values and NOEs for Yariv reagent in DMF.[a]



The yariv reagent, R is β-Glc or α -Gal

Carbon	β-Glc Yariv d (ppm)	NT_1 (s)	NOE	α-Gal Yariv d (ppm)	NT_1 (s)
C1[b]	100.30	0.20	1.85	98.61	0.30
C2	73.25	0.19	1.94	68.70	0.28
C3	77.08	0.21	1.84	69.66	0.28
C4	69.69	0.24	1.71	68.26	0.24
C5	76.57	0.24	1.76	72.12	0.26
C6	60.71	0.48	1.90	60.53	0.64
Ph2,4,6[c]	118.47	0.20	1.68	117.94	0.16
Ph1,3,5[c]	117.41	0.20	1.76	117.71	0.20

The Yariv reagent formula is also presented.
[a] Shifts were measured relative to the solvent peak at 30.1 ppm and are expressed relative to ppm.
[b] C1–C6 refer to the sugar carbons.
[c] Ph2,4,6 and Ph1,3,5 refer to the phenolic ring carbons.

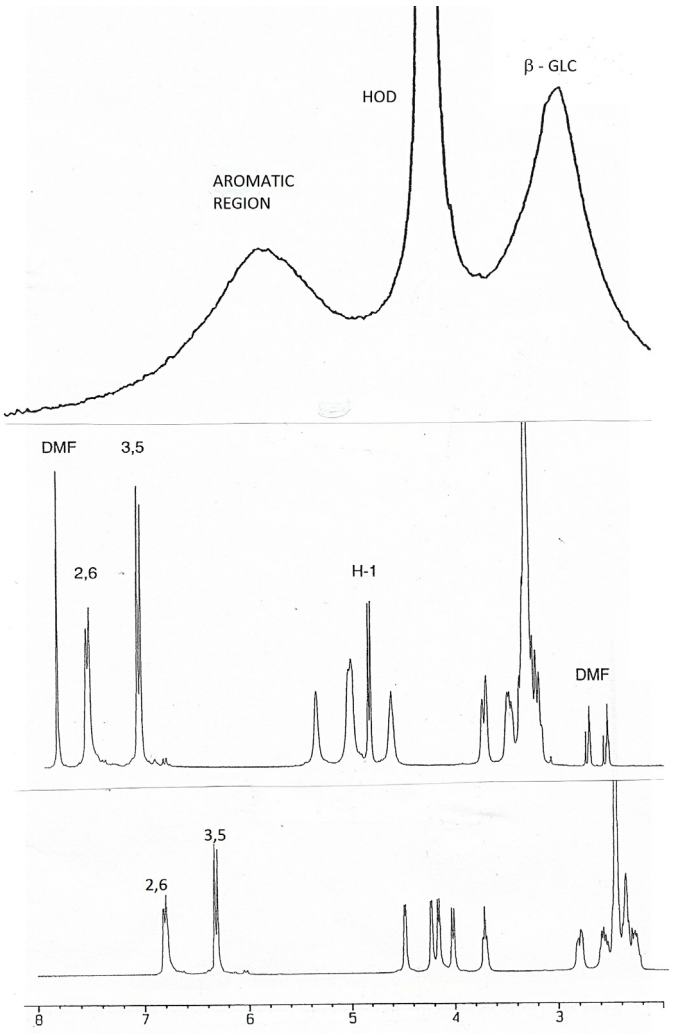


Fig. 1. 300 MHz ^1H NMR spectrum of β-D-Glc Yariv in D_2O (upper), DMF (middle) and DMSO (lower).

The AGPs to which Yariv reagents bind and precipitate are a complex family of ubiquitous plant proteoglycans found at the cell surface (plasma membrane and cell wall) and in secretions, that are of enormous interest due to their proposed biological functions (reviewed by Ellis, Egelund, Schultz, & Bacic, 2010; Gaspar et al., 2001; Nothnagel, 1997; Seifert & Roberts 2007; Showalter, 2001) and commercial utility as emulsifiers widely used in the food industry.

Recently, Tan et al. (2013) demonstrated that AGPs are key crosslinking molecules within the cell wall of *Arabidopsis*. Their work provides experimental evidence for an AGP covalent complex, ARABINOXYLANPECTIN ARABINOGALACTAN-PROTEIN 1 (APAP1) that crosslinks the major wall network of non-cellulosic polysaccharides and pectins. They belong to the hydroxyproline-rich superfamily of cell wall glycoproteins (HRGPs; Johnson et al., 2003) and are composed primarily of carbohydrate (90–98% w/w) attached to a polypeptide backbone rich in the amino acids Hyp/Pro, Ala and Ser that has a glycosylphosphatidyl-inositol (GPI) membrane anchor attached to the C-terminus.

The diversity of polypeptide backbones has resulted in a classification of AGPs based on motifs and modules within the protein backbones (Johnson et al., 2003). The carbohydrate is primarily in the form of type 11 arabinogalactan (AG) polysaccharide chains attached to Hyp on the protein backbone that are (1 → 3)-β-D-Gal*p* polymers substituted by (1 → 6)-β-D-Gal*p* chains. Both the 3-linked and 6-linked Gal residues may be substituted by Ara*f* residues, and depending on the source, other monosaccharides may also be substituents (Anderson, Clarke, Jermyn, Knox, & Stone, 1977; Fincher, Sawyer, & Stone, 1974; Samuelsen et al., 1995). Short arabinooligosaccharide chains are also found attached to the polypeptide backbone (see Fig. 1 in Ellis et al., 2010). Advances in sequencing technologies have enabled the fine structure of the glycan moieties of AGPs to be determined in synthetic fusion glycoproteins (Tan et al., 2010), wheat flour AG peptide (Tryfona et al., 2010) and *Arabidopsis* leaf AGP (Tryfona et al., 2012) and these provide higher resolution structures based upon the generally accepted core backbone structures.

Little is known about the structure of the site on AGPs to which the β-D-Glc Yariv reagent binds. Conventional glycoside haptens do not compete for binding, although some flavonol glycosides present in crude tissue extracts may prevent binding between AGPs and the β-D-Glc Yariv reagent (Jermyn, 1978b). There is some evidence that the binding site on AGPs is in a Hyp-rich domain, and that it involves both the protein backbone as well as part of the galactan backbone of the AG (Gleeson & Jermyn, 1979; Jermyn & Yeow, 1975). Enzymic digestion of AGP with subtilisin removed over 80% of the protein but left a Hyp-rich core which retained the capacity to bind β-D-Glc Yariv reagent (Gleeson & Jermyn, 1979). AGs (protein free) from larch (*Larix decidua*) and the Hyp-containing AG-peptide from *Lolium multiflorum* do not bind the Yariv reagent, suggesting the need for some protein in the binding. Surprisingly, a carrot (*Daucus carota*) AGP which lacks Hyp binds the β-D-Glc Yariv reagent (Baldwin, Mc Cann, & Roberts, 1993). In an exciting new development Kitazawa et al. (2013) conducted a series of experiments that concluded that β-Glc Yariv binds specifically to (1 → 3)-β-D-galacto-oligosaccharides of DP > 5 but not to (1 → 6)-β-D-galacto-oligosaccharides. However, their findings are not always consistent with those described above where the protein component is important for binding. It is also interesting to note that the polysaccharide fractions isolated from the Malian

medicinal tree (*Terminalia macroptera*) show differing abilities to form precipitates with the Yariv reagent although containing 3,6-linked Gal. The relative amounts of 3-linked Gal for the two that precipitate with Yariv reagent is greater than for the two that do not precipitate. The two fractions that precipitate with Yariv are also devoid of protein (Zou et al., 2014).

The role of terminal Ara*f* residues in the interaction is seemingly complex. Both Gleeson, Jermyn, and Clarke (1979) and Akiyama and Kato (1981) showed that the progressive removal of terminal Ara*f* residues by acid hydrolysis led to a loss in complexing ability. Treatment of rose (*Rosa glauca*) AGP with 12.5 M oxalic acid at 100 °C for 5 h led to a loss in 3,6-linked Gal with a parallel increase in 6-linked Gal and resulted in a product with a greatly diminished interaction with the β-D-Glc Yariv reagent (Komalavilas, Zhu, & Nothnagel, 1991). In contrast to these observations, the specific removal of Ara*f* residues by α-L-arabinofuranosidase action on the *Angelica acutiloba* AGP (Kiyohara, Cyong, & Yamada, 1989) increased the interaction and this was accompanied by an increase in 6-linked (un-substituted) Gal residues. However, complete removal of carbohydrate with either anhydrous HF or progressive acid hydrolysis of AGPs with 0.1–1 M HCl, led to loss of binding (Gleeson & Jermyn, 1979). In contrast, Kitazawa et al. (2013) found that enzymic removal of terminal α-L-*Araf* and β-D-GlcA*p* residues on (1 → 6)-β-D-Gal side chains had no impact of β-Glc Yariv binding to radish (*Raphanus*) root AGP.

In this paper we report the results of a study on the aggregation state and conformation of β-D-Glc and α-D-Gal Yariv reagents in aqueous and organic solvents using NMR and dynamic light scattering methods. The interactions of the β-D-Glc Yariv reagent with a fraction from a gum arabic (*Acacia senegal*) AGP separated by hydrophobic interaction chromatography (Osman, Menzies, Williams, Phillips, & Balwin, 1993) and the products from one fraction treated with dilute acid is also reported.

2. Experimental

2.1. Preparation of the Yariv reagents

The β-D-glucosyl (Glc)-Yariv reagent [1,3,5-tri (4-β-D-glucopyranosyl-oxyphenylazo)-2,4,6-trihydroxybenzene] was synthesised according to Yariv et al. (1962) with minor modifications. The α-D-galactosyl (Gal) Yariv reagent was prepared by the late Dr. Michael Jermyn according to Yariv et al. (1962).

2.2. NMR spectroscopy

All ^{1}H and ^{13}C NMR spectra were recorded at 300 and 75 MHz, respectively, on a Bruker AM wide-bore spectrometer. The spectra were recorded using a 5 mm NMR tube with spectral widths of approximately 3000 Hz and 15 000 Hz for ^{1}H and ^{13}C spectra, respectively. The proton decoupled ^{13}C spectra employed WALTZ-16 broad-band decoupling in order to minimise solvent heating effects. The ^{13}C T_1 spectra were measured using the inversion recovery Fourier transform method with a set of twelve τ values, recorded in interleaved mode, with cycling in blocks of 16 transients. The ^{13}C T_1 values were calculated by exponential fitting of the raw signal intensity data using standard Bruker software. ^{13}C NOE values were measured from the ratio of resonance intensities from a spectrum with continuously applied WALTZ-16 decoupling (maximum possible NOE effect) and a spectrum with the WALTZ-16 decoupling gated on only during acquisition (no NOE effect). The relaxation and NOE data listed are an average of three experiments, with an estimated error of less than 10%.

300 MHz ^{1}H NMR spectra were recorded on the β-Glc Yariv reagent in a number of solvents and at several concentrations. In most studies the concentration was approximately 10 mg/ml, a value much higher than that used for AGP precipitation. This was necessitated by the relatively low sensitivity of the NMR technique for the ^{13}C natural abundance relaxation experiments. Both ^{1}H and ^{13}C NMR spectra were recorded in D_2O, DMF, DMSO and mixtures of D_2O and DMF. A parallel series of experiments was conducted using α-Gal Yariv reagent.

2.3. Absorption spectra

Spectra were recorded using a Cary 1 spectrophotometer (Varian) in the range 220–600 nm. The concentrations of β-Glc- and α-Gal-Yariv reagents were 15 mg/ml in all experiments. The absorption spectra were recorded in water, 1% NaCl, 10% NaCl, 8 M urea and SDS (0.1–6 g/l).

2.4. Quasi elastic light scattering

QELS measurements were made using a Malvern 4700 light scattering system at 90° using a 100 mW Ar^{+} ion laser operating at 488 nm and a power of 10 mW. The correlation functions were analysed using the CONTIN algorithm. The diffusion coefficients, thus obtained, were converted to hydrodynamic radii using the Stokes–Einstein equation (Berne & Pecora, 1976). As the solutions of the Yariv reagents show significant absorption at 488 nm, the absorption of scattered radiation was minimised by using the smallest cell available (3 mm i.d.). Samples were normally 1 mg/ml. No difference in the measured size was observed on dilution of the samples, or for the different cell sizes of 3, 8 and 25 mm diameter indicating that the absorption did not affect the measured size. This also indicates that the samples were dilute and therefore no interaction effects were present.

2.5. Preparation of arabinogalactan-protein (AGP) fractions from gum acacia

Gum acacia powder (BP 1AS, Batch No. PUR490) was obtained from Woods & Woods Pty. Ltd. Auburn, New South Wales, Australia. The powder (7 g) was dissolved by boiling in distilled water and filtered through glass fibre filter paper (Whatman GF/C) prior to fractionation by chromatography on Phenyl-Sepharose CL-4B as described by Osman et al. (1993). Four fractions were obtained, Fr.1A and Fr.1B by elution with 4.2 M NaCl, Fr.2 by elution with 2 M NaCl and Fr.3 by elution with distilled water.

2.6. Oxalic acid hydrolysis of Fr.2 from gum acacia

Freeze dried Fr.2 (2 mg/ml) was hydrolysed at 100 °C with 12.5 mM oxalic acid. Aliquots (1 ml) were withdrawn at 0.5, 1, 2, 4 and 6 h, and neutralised immediately with 1 ml 12.5 mM NH_4HCO_3. Portions of the neutralised hydrolysates (1 ml) were desalted on a PD10 column (Pharmacia). The desalted samples were tested for their interaction with the β-Glc Yariv reagent and analysed for their monosaccharide, linkage and amino acid compositions.

2.7. Single radial gel-diffusion of polysaccharides using the Yariv reagents

The AGPs were tested for reactivity against the β-D-Glc Yariv reagents by single gel diffusion (Van Holst & Clarke, 1985). α-D-Gal-Yariv reagent was used as a control. Samples (normally 20 μg) were applied in wells of agarose (1%) plates containing the Yariv reagent (10 or 20 μg/ml) and incubated overnight at room temperature.

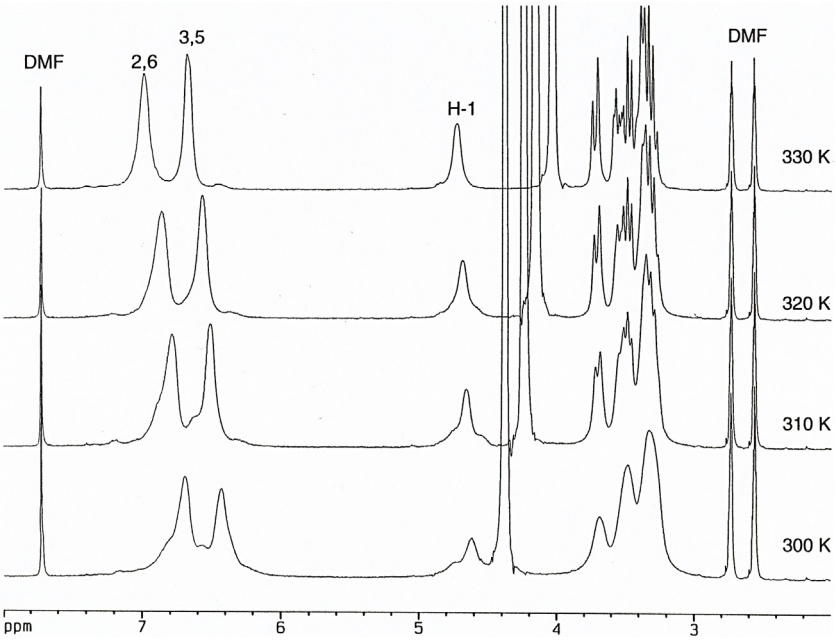


Fig. 2. 300 MHz ^{1}H NMR spectrum of β-D-Glc Yariv in 50% DMF in D_2O at different temperatures.

2.8. Monosaccharide analysis

Aliquots of the desalted AGP samples were converted to their methyl glycosides by the method of Chaplain (1982). The resulting TMS-derivatives of the methyl glycosides were analysed by GC–MS as described by McConville, Homans, Thomas-Oates, Dell, and Bacic (1990).

2.9. Carboxyl reduction of uronic acids

Reduction of the uronic acids was performed using the method of Kim and Carpita (1992) essentially as described by Sims and Bacic (1995).

2.10. Linkage analysis by methylation

The carboxyl-reduced samples were methylated by the NaOH procedure of Ciucanu and Kerek (1984) essentially as described by McConville et al. (1990). The partially methylated alditol acetates were analysed by GC–MS using the method of Lau and Bacic (1993).

3. Results and discussion

3.1. Solution state properties of the Yariv reagents

3.1.1. NMR

The 300 MHz ^{1}H NMR spectrum recorded on the β-D-Glc Yariv reagent (Fig. 1) shows that in D_2O the spectral lines for the aromatic and aliphatic protons are extremely broad (the sharpest peak in the spectrum is due to the residual HOD signal from the D_2O). Individual resonances within the two spectral regions are not resolvable and a single broad envelope is observed, each for the aromatic and aliphatic protons. The line widths at half-peak height of these envelopes are approximately 500 Hz for the aromatic resonances and 280 Hz for the aliphatic resonances. The broad peaks are characteristic of very large aggregates. By contrast, the spectrum of β-D-Glc Yariv reagent in DMF (Fig. 1) shows sharp peaks characteristic of a monomeric species. A similar trend is seen in the DMSO spectrum (Fig. 1). These results confirm previous reports (Woods et al., 1978) that the Yariv reagent has an extremely high tendency to aggregate in aqueous media but is completely dissociated in organic solvents.

In additional experiments, the effects of various excipients and heating on the aggregation state in aqueous solutions were determined (Fig. 2). When the Yariv reagents were dissolved in 10% NaCl there was no change to the spectrum relative to D_2O (data not shown). In 8 M urea and 6.8 M guanidinium HCl there was, however, some sharpening of the broad aliphatic and aromatic resonances. In 8 M urea, the line widths decreased by a factor of 2–2.5 from those in pure D_2O, and for 6.8 M guanidinium HCl the reduction in line widths was slightly greater (Fig. 3). In both cases, it became possible to discern some sub-components within the peak envelopes, especially in the aliphatic region. Increasing the temperature resulted in a sharpening of the resonances of a 50% DMF solution of β-D-Glc Yariv reagent, consistent with a reduction in aggregation at the higher temperatures (Fig. 2). The degree of disruption of the aggregates caused by a 30 °C increase in temperature is roughly equivalent to that seen in a DMF solution compared to pure D_2O (see later). Dilution of aqueous samples by a factor of 2 did not produce a significant reduction in line width.

The spectra obtained when DMF was titrated into a D_2O solution of the Yariv reagent are shown in Fig. 4. Successive addition of DMF results in significant and progressive sharpening of the resonances. The degree of disaggregation caused by the addition of even a moderate percentage of DMF is significantly greater than that caused either by raising the temperature or the presence of high concentrations of either urea or guanidinium HCl. These results suggest that the Yariv reagent aggregates by both hydrophobic interactions and hydrogen bonding. In solutions containing high concentrations of urea hydrogen bonding between the β-D-Glc Yariv reagent molecules will be disrupted, but hydrophobic interactions may still be present, causing stacking, but in a way different from that found in pure water. In DMF and DMSO neither type of interaction would be effective and a solution of monomers results.

An attempt was made to record a ^{13}C spectrum of β-Glc Yariv in D_2O, however no signals were detected, presumably because resonances were significantly broadened. The ^{13}C spectra of β-D-Glc Yariv in DMF and DMSO show, as expected, sharp signals characteristic of monomeric species, consistent with the findings from the ^{1}H NMR spectra. The signals were assigned based on the ^{13}C

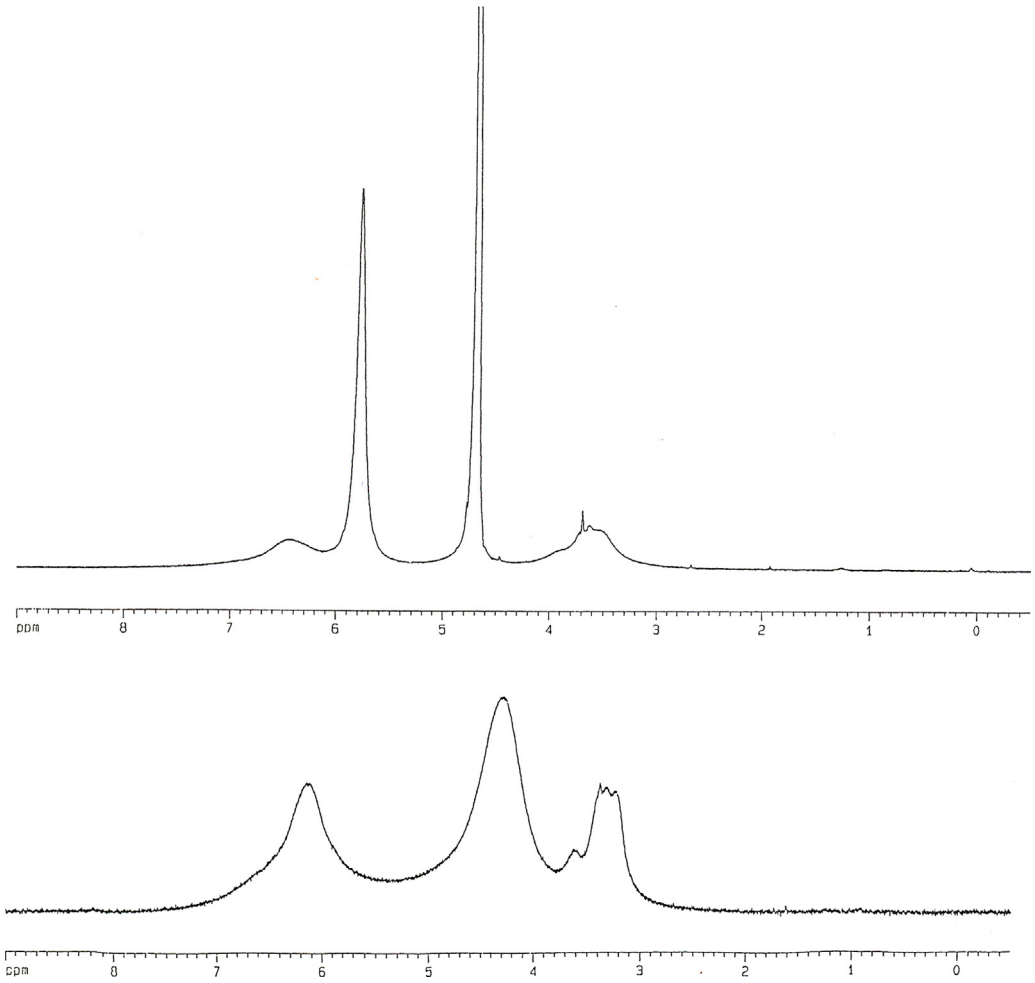


Fig. 3. 300 MHz ^{1}H NMR spectrum of β-D-Glc Yariv in 8 M urea (upper) and 6.8 M guanidine HCl (lower).

spectra of model compounds and from a 2D HMQC spectrum and are reported in Table 1, as are a series of spin-lattice relaxation time measurements recorded to characterise the mobility of the Yariv reagent in DMF. In general, an increase in NT_1 (where N is the number of attached protons) reflects an increase in molecular motion (Craik, Levy, & Kumar, 1983). As the NT_1 values for all the protonated carbons are similar (ca. 0.20 s) this suggests that they all undergo similar motion, i.e. the β-D-Glc Yariv reagent has no significant degree of internal flexibility. Heteronuclear ^{13}C(^{1}H) NOEs were also measured and these ranged from 1.68 to 1.94 (Table 1). The reduction of these NOEs from the theoretical maximum of 2.98, expected for rapidly tumbling organic molecules, confirms that there is a significant degree of motional restriction, even in organic solvents.

When the NT_1 and NOE data were fitted to the appropriate equations (Craik et al., 1983, Levy et al., 1980) relating relaxation data and correlation times it was found that a motional model involving isotropic tumbling in solution was inconsistent with the experimental data as such a model would require a correlation time of 0.8 ns to fit the NOE data and a value of 0.2 ns to fit the T_1 data. This result strongly suggests that tumbling of the Yariv reagent in solution is anisotropic, as might be expected from the shape of the molecule. The flat aromatic framework with pendant sugar rings (Fig. 1) predisposes the molecule to faster relative rotation about an axis perpendicular to the aromatic core rather than parallel with it.

The results of a parallel series of experiments with the α-D-Gal Yariv analogue, in general, indicated that this compound has a lower tendency to aggregate than the β-D-Glc Yariv analogue. In particular, the spectral lines in aqueous solution, while still extremely broad, are a factor of three sharper than those for the β-D-Glc Yariv reagent. The anomeric proton of the α-D-Gal is also visible. This observation suggests that there is some discrimination of sub-peak components within the aliphatic and aromatic peak envelopes. For the α-D-Gal Yariv reagent, the ^{13}C T_1 values are, on average, larger than for β-D-Glc Yariv (Table 1) and are spread over a larger range. This trend indicates that there is a significantly greater degree of both overall and internal flexibility of the α-D-Gal Yariv reagent compared to the β-D-Glc Yariv reagent.

3.1.2. Light scattering

The light scattering radii of 82 and 122 nm for the α-D-Gal Yariv and β-D-Glc Yariv reagents, respectively, in water are consistent with the data obtained from the NMR studies indicating rather large aggregates for both molecules (Table 2). The DMF data supports the conclusion from NMR that the Yariv reagents lose their ability to aggregate and exist as solubilised single molecules in the organic solvent as is consistent with predominantly hydrophobic molecules. Furthermore, with increasing salt concentration in water the hydrodynamic radii decreased, but not to the same extent as in the presence of DMF. The size of the β-D-Glc Yariv reagent aggregates appears to be similar in 10% NaCl and 8 M urea (Table 2).

3.1.3. Absorption spectra

The absorption spectra (Fig. 5) are consistent with the interpreted degree of aggregation in the various solvents. The spectra

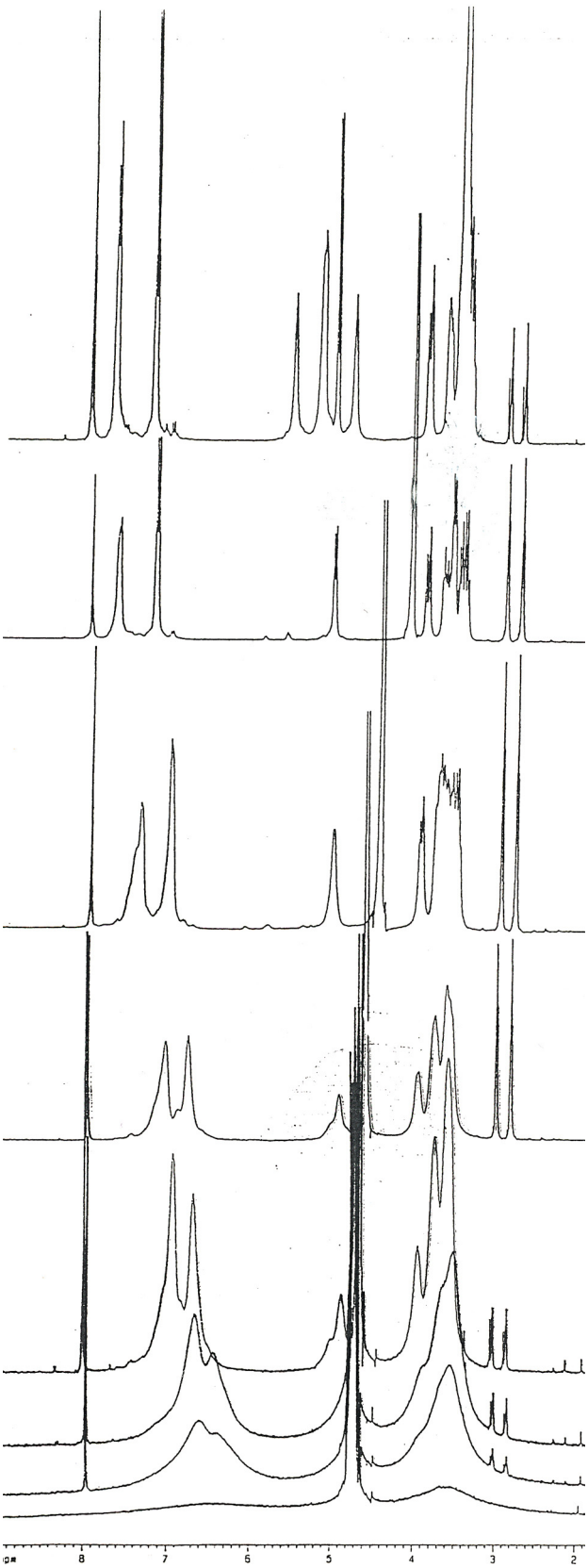


Fig. 4. 300 MHz ^{1}H NMR spectrum when DMF is titrated into a D_2O solution of β-D-Glc Yariv. Top, DMF, then D_2O/DMF ratios 1:6, 3:6, 5:6, 6:5, 6:2, 6:1, bottom D_2O.

Table 2
Light scattering measurements of hydrodynamic radii of Yariv reagents in various solvents and calculated aggregate sizes.

Solvent	β-Glc Yariv Radius (nm)	α – Gal Yariv Radius (nm)
H_2O	122	82
50%DMF	1.5	1.5
100%DMF	0.4	0.5
1%NaCl	74	n.d.
10%NaCl	50	n.d.
8 M urea	52	n.d.
Calc. aggregate size in H_2O	305	164

n.d. = not determined.

show the same trends as those observed by Woods et al. (1978). The absorption peak at 512 nm is due to the monomer whereas the peak at 407 nm indicates the presence of aggregates. The spectral shift to lower wavelength (higher energy) in the aggregated form is due to the presence of aggregates where excitation interaction occurs (Evans & Bohn, 1993; Marowsky & Steinhoff, 1988; Schildkraut, Penner, Willand, & Ulman, 1988). This indicates that the chromophores of the Yariv complex interact in the aggregate. The spectra of the β-D-Glc Yariv reagent in DMF is of interest in that an intermediate state is present, possibly a dimer, which shows an absorption maximum at 460 nm. The spectra also show an increasing degree of dissociation of the aggregate with increasing SDS concentration up to 20 g/l.

3.1.4. Conclusion on solution state properties of the Yariv reagents

NMR and light scattering experiments show that the β-D-Glc Yariv reagent is aggregated in D_2O and that the aggregate probably consists of approx. 305 units, given that the hydrodynamic radius is the same for one unit whether it is stacked or not. In 1% NaCl the number of aggregated molecules is approximately 185 and this decreases to approximately 125 in 10% NaCl. It is interesting to note that complexation between AGP and β-D-Glc Yariv reagent in 1% NaCl can occur but is disrupted in 10% NaCl, suggesting that the β-D-Glc Yariv reagent aggregate must comprise some 100–150 units to complex with the AGP. In 8 M urea the size of the aggregate is approximately the same as in 10% NaCl. The NMR spectra in D_2O and 10% NaCl are identical, but in 8 M urea and 6.8 M guanidinium HCL both the aliphatic and aromatic resonances are sharpened. Hydrogen bonding appears to be disrupted more

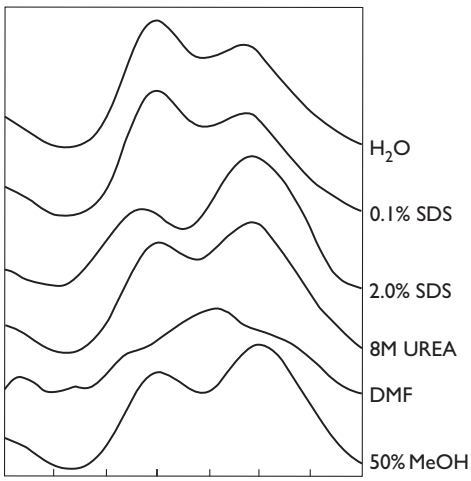


Fig. 5. Absorption spectra of β-D-Glc Yariv in different solutions.

Table 3
Changes in monosaccharide compositions and linkages of gum Arabic Fr.2 AGP after different times of 12.5 M oxalic acid hydrolysis at 100 °C.

(A) Relative monosaccharide composition of gum Arabic Fr.2 AGP subjected to different times of 12.5 M oxalic acid hydrolysis at 100 °C

Monosaccharide	Start	0.5 h	1 h	2 h	4 h	6 h
Ara	33	29	24	16.5	8	8
Rha	12	13	12	9	4	3
Gal	45	46	49	58	69	67
GlcA	10	12	15	16.5	19	22

(B) Ratio Arabinose to galactose of gum Arabic Fr.2 AGP subjected to different times of 12.5 M oxalic acid hydrolysis at 100 °C

Ratio	Start	0.5 h	1 h	2 h	4 h	6 h
Ara/Gal	0.73	0.44	0.49	0.28	0.12	0.12
Rha/Gal	0.27	0.27	0.24	0.16	0.06	0.04
GlcA/Gal	0.22	0.26	0.30	0.28	0.27	0.32

(C) Monosaccharide composition (μg) of gum Arabic Fr.2 AGP subjected to different times of 12.5 M oxalic acid hydrolysis at 100 °C

Monosaccharide	Start	0.5 h	1 h	2 h	4 h	6 h
Ara	81	45	31	18	6	5
Rha	29	21	15	10	3	2
Gal	110	72	62	63	50	40
GlcA	25	19	18	18	13	13
Total	245	157	126	109	72	60

(D) Mol% of the different types of linkages for the monosaccharides present in gum Arabic Fr.2 AGP after oxalic acid hydrolysis for different periods

Type of linkage	Start	0.5 h	1 h	2 h	4 h	6 h
t-Ara*f*	18.5	18.5	16.2	8.3	1.2	1.2
t-Ara*p*	1.1	1.1	1.1	1.1	1.2	1.2
3-Ara*f*	6.5	6.5	4.2	2.1	1.2	1.2
5-Ara*f*	10.7	6.5	5.4	6.5	5.4	5.4
t-Rha*p*	13.3	14.3	13.3	10	4.7	3.3
t-Gal*p*	14	12	16.2	16.1	21.9	23
3-Gal*p*	2.2	3	3.6	4.5	7.7	5.8
6-Gal*p*	2.2	5.5	9.1	16.5	22.2	24
3.6-Gal*p*	11	13.6	12	12.9	16.1	13.5
3,4,6-Gal*p*	11	8.1	4.9	3.9	Tr.	Tr.
T-GlcA*p*	1	1	2	2.9	6.1	8.1
4-GlcA*p*	8.1	10	12	15.2	12.4	13.2

than the hydrophobic interactions, and thus hydrogen bonding is an important factor involved in aggregation in aqueous solution. In the organic solvents, DMF and DMSO, both the NMR and light scattering experiments showed that the β-D-Glc Yariv reagent was present as monomers. When titrating a D_2O solution of the β-D-Glc Yariv reagent with DMF there is a marked sharpening of the resonances even after a small addition of DMF most likely caused by disruption of both the hydrophobic interaction in the aromatic region and the hydrogen bonds in the carbohydrate region. It is of interest to note that the aggregate in D_2O/DMF (1:1) comprised only 3 β-D-Glc Yariv molecules (Table 2). These results confirm those of Woods et al. (1978) concerning aggregate formation in different media, but the size of the aggregate in water appears to be much larger than was suggested by those workers based upon ultracentrifugation. It is also of interest that the disruption of the AGP-Yariv complex takes place in conditions when the β-D-Glc Yariv reagent is relatively highly aggregated.

3.2. Interaction of Yariv reagent with AGPs and modified AGPs

3.2.1. Characterisation of AGP and the hydrolysis products

Gum acacia AGP was fractionated by hydrophobic interaction chromatography as described by Osman et al. (1993) into four fractions that all precipitated the β-D-Glc Yariv reagent. The fraction eluted with 2 M NaCl (Fr.2) was chosen for further studies on the β-D-Glc Yariv–AGP interaction. This polymeric material was partially hydrolysed with oxalic acid (12.5 M, 100 °C). Samples withdrawn at different hydrolysis time intervals (0–6 h) were tested for their ability to bind the β-D-Glc Yariv reagent and analysed for monosaccharide and linkage composition.

Table 3 summarises the relative changes between the monosaccharides present in the samples after the different time periods of hydrolysis. Ara and Rha are lost to a great extent, starting approximately 0.5–1 h after hydrolysis. In contrast, the proportions of both Gal and GlcA increase during the time course of hydrolysis whereas the ratio between Gal and GlcA stays constant. The linkage composition of the monosaccharides present after the different oxalic acid hydrolysis periods is given in Table 3D. Gum acacia Fr.2 is highly branched with Gal*p* the only branched sugar with 3,6- and 3,4,6-Gal*p* predominating. During hydrolysis most of the Ara*f* units are lost, only minor amounts are still present after 6 h of hydrolysis. The small amount of Ara*p* is not, as expected, removed with the conditions used. Rha*p*, present as terminal units only, is progressively lost during the hydrolysis, and after 4 h little is left.

GlcA*p*, originally mainly 4-linked with a small amount of terminal, has lost substituents through the experimental period as the ratio between the terminal and 4-linked units changed from 1:8 to 1: 1.6. There is also an increase in the relative amount of 6-Gal*p* throughout the experiment with a concomitant total loss of 3,4,6-Gal*p*, while the amount of 3,6-Gal*p* stays almost unchanged; 3-Gal*p* is unchanged. This indicates that the backbone of the galactan-part of the molecule is 6-linked, and the loss is on C-3 of the linked units. Table 3C is showing the real amounts of loss during the hydrolysis process. Ara and Rha are lost almost completely, while approximately 1/3 of Gal is left. Thus the 3-linked Gal chains have probably been reduced to only a few units.

3.2.2. Interaction of AGP and degradation products with the Yariv reagent

The ability of the hydrolysis products to precipitate the β-D-Glc Yariv reagent did not change during the first hour of hydrolysis; the reaction weakened somewhat after 2 h, and after 4 h the ability to bind the Yariv reagent was lost completely.

To ensure that this was a real loss, and not a decrease in ability to react, a similar experiment was performed with double the amount of the β-D-Glc Yariv reagent in the gel. No precipitation rings were obtained for samples withdrawn after 4 and 6 h acid hydrolysis, indicating that the binding ability was lost.

The main compositional differences between the samples withdrawn after 2 and 4 h are the loss of Ara and Rha with a relative increase of Gal and GlcA. Structurally the relative loss of branched Gal-units is the most prominent and also the reduced content of Ara.

3.2.3. Conclusion on the interaction of the Yariv reagent with AGPs and modified AGPs

The results obtained in this study are similar to those obtained on the AGP from rose (Komalavilas et al., 1991), but differ from those performed on the *A. acutiloba* AGP (Kiyohara et al., 1989), where it was found that an increase in 6-Gal*p* units gave an increase of the ability to bind the Yariv reagent. Recently Kitazawa et al. (2013) found on radish root AGPs that removal of terminal α-L-Ara*f* and β-D-GlcA*p* residues on (1 → 6)-β-D-Gal side chains had no impact on β-D-Glc Yariv binding. The results obtained in that paper (Kitazawa et al., 2013) also show that the Yariv reagent will bind to galacto-oligosaccharides with 1,3 linkages if they are of a certain size, i.e. minimum 5–7 units.

Focusing on the Gal units and the changes in their linkage types during the hydrolysis process (i.e. the change in the amounts of different types of linkages), it appears that the amount of 3-linked Gal is substantially reduced during the course of the acid hydrolysis. Thus we can conclude, as did Kitazawa et al. (2013), that a certain length of 3-linked Gal residues is necessary for Yariv binding. Therefore our results suggest that it is a relative increase in 6-Gal residues, concomitant with a decrease of 3-Gal residues that could be resulting in shorter 3-Gal regions as the hydrolysis proceeds.

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References

Akiyama, K., & Kato, K. (1981). An extracellular arabinogalactanprotein from *Nicotiana tabacum*. *Phytochemistry*, *20*(11), 2507–2510.

Anderson, R. L., Clarke, A. E., Jermyn, M. A., Knox, R. B., & Stone, B. A. (1977). A carbohydrate-binding arabinogalactan-protein from liquid suspension cultures of endosperm from *Lolium multiflorum*. *Australian Journal of Plant Physiology*, *4*, 143–158.

Baldwin, T. C., Mc Cann, M. C., & Roberts, K. (1993). A novel hydroxyproline deficient arabinogalactan protein secreted by suspension-cultured cells of *Daucus carota*. *Plant Physiology*, *103*, 115–123.

Berne, B. J., & Pecora, R. (1976). Dynamic light scattering with application to chemistry. *J. Wileys & Sons*.

Chaplain, M. F. (1982). A rapid and sensitive method for the analysis of carbohydrate components in glycoproteins using gas–liquid chromatography. *Analytical Biochemistry*, *123*, 336–341.

Ciucanu, I., & Kerek, F. (1984). A simple and rapid method for the permethylation of carbohydrates. *Carbohydrate Research*, *131*, 209.

Craik, D. J., Levy, G. C., & Kumar, A. (1983). MOLDYN: A generalized program for the evaluation of molecular dynamics using nuclear magnetic resonance spin-relaxation data. *Journal of Chemical Information and Computer Science*, *23*, 30–38.

Ellis, M., Egelund, J., Schultz, C., & Bacic, A. (2010). Arabinogalactan-proteins (AGPs): Key regulators at the cell surface? *Plant Physiology*, *153*, 403–419.

Evans, C. E., & Bohn, P. J. (1993). Characterization of an aggregate-sensitive single-component energy-transfer system. *Journal of American Chemical Society*, *115*, 3306.

Fincher, G. B., Sawyer, W. H., & Stone, B. A. (1974). Chemical and physical properties of an arabinogalactan-peptide from wheat endosperm. *Biochemical Journal*, *139*, 535–545.

Gane, A. M., Craik, D., Munro, S., Howlett, L. A., Clarke, G. J., & Bacic, A. E. A. (1995). Structural analysis of the carbohydrate moiety of arabinogalactan-proteins from stigmas and styles of *Nicotiana alata*. *Carbohydrate Research*, *277*, 67–85.

Gaspar, Y., Johnson, K. L., McKenna, J. A., Bacic, A., & Schultz, C. J. (2001). The complex structures of arabinogalactan-potein and the journey towards understanding function. *Plant Molecular Biology*, *47*, 161–176.

Gleeson, P. A., & Jermyn, M. A. (1979). Alteration in the composition of β-lectins caused by chemical and enzymic attack. *Australian Journal of Plant Physiology*, *6*, 25–30.

Gleeson, P. A., Jermyn, M. A., & Clarke, A. A. E. (1979). Isolation of an arabinogalactan protein by lectin affinity chromatography on tridacnin-sepharose 4B. *Analytical Biochemistry*, *92*, 41–45.

Jermyn, M. A. (1978a). Comparative specificity of concanavalin A and the β-lectins. *Australian Journal of Plant Physiology*, *5*, 687–696.

Jermyn, M. A. (1978b). Isolation from the flowers of *Dryandra praernosa* of a flavonol glycoside that reacts with β-lectins. *Australian Journal of Plant Physiology*, *5*, 697–705.

Jermyn, M. A., & Yeow, Y. M. (1975). A class of lectins present in the tissues of seed plants. *Australian Journal of Plant Physiology*, *2*, 501–531.

Kim, J.-B., & Carpita, N. C. (1992). Changes in esterification of the uronic acid groups of cell wall polysaccharides during elongation of maize coleoptiles. *Plant Physiology*, *98*, 646.

Kitazawa, K., Tryfona, T., Yoshimi, Y., Hayashi, Y., Kawauchi, S., Antonov, L., et al. (2013). β-Galactosyl Yariv reagent bind to β-1,3-galactan of arabinogalactan proteins. *Plant Physiology*, *161*, 1117–1126.

Kiyohara, H., Cyong, J.-C., & Yamada, H. (1989). Studies on polysaccharides from *A. acutiloba*. Part XI. Relationship between structure and activity of an anti-complementary arabinogalactan from the roots of Angelica acutiloba Kitagawa. *Carbohydrate Research*, *193*, 193–200.

Komalavilas, P., Zhu, J.-K., & Nothnagel, E. A. (1991). Arabinogalactan-proteins from the suspension culture medium and plasma membrane of rose cells. *Journal of Biological Chemistry*, *266*, 15956–15965.

Lau, E., & Bacic, A. (1993). Capillary gas chromatography of partially methylated alditol acetates on a high-polarity, cross-linked, fused-silica BPX70 column. *Journal of Chromatography*, *637*, 100–103.

Marowsky, G., & Steinhoff, R. (1988). Hemicyanine monolayer orientation studied by second-harmonic generation. *Optics Letters*, *13*, 707–709.

McConville, M. J., Homans, S. W., Thomas-Oates, J. E., Dell, A., & Bacic, A. (1990). Structures of the glycoinositolphospholipids from *Leishmania major*. A family of novel galactofuranose-containing glycolipids. *Journal of Biological Chemistry*, *265*, 7385–7394.

Nothnagel, E. A. (1997). Proteoglycans and related components in plant cells. *International Review of Cytology*, *174*, 195–291.

Nothnagel, E., & Lyon, J. L. (1986). Structural requirements for the binding of phenylglycosides to the surface of protoplasts. *Plant Physiology*, *80*, 91–98.

Osman, M. E., Menzies, A. R., Williams, P. A., Phillips, G. O., & Balwin, T. C. (1993). The molecular characterisation of the polysaccharide gum from *Acacia senegal*. *Carbohydrate Research*, *246*, 303–318.

Samuelsen, A. B., Paulsen, B. S., Wold, J. K., Otsuka, H., Yamada, H., & Espevik, T. (1995). Isolation and partial characterization of biologically active polysaccharides from *Plantago major* L. *Phototherapy Research*, *9*, 211–218.

Schildkraut, J. S., Penner, T. L., Willand, C. S., & Ulman, A. (1988). Absorption and second-harmonic generation of monomer and aggregate hemicyanine dye in Langmuir-Blodgett films. *Optics Letters*, *13*, 134–136.

Schopfer, P. (1990). Cytochemical identification of arabinogalactan protein in the outer epidermal wall of maize coleoptiles. *Planta*, *183*, 139–142.

Seifert, G. J., & Roberts, K. (2007). The Biology of Arabinogalactan Proteins. *Annual Review of Plant Biology*, *58*, 137–161.

Showalter, A. M. (2001). Arabinogalactan-proteins: Structure, expression and function. *Cellular and Molecular Life Science CMLS*, *58*, 1399–1417.

Sims, I. M., & Bacic, A. (1995). Extracellular polysaccharides from suspension cultures of Nicotiana plumbaginifolia. *Phytochemstry*, *38*, 1397–1405.

Tan, L., Eberhard, S., Pattathil, S., Warder, C., Glushka, J., Yuan, C., et al. (2013). An Arabidopsis cell wall proteoglycan consists of pectin and arabinoxylan covalently linked to an arabinogalactan protein. *Plant Cell*, *25*, 270–287.

Tan, L., Varnal, P., Lamport, D. T. A., Yuan, C., Xu, J., Qiu, F., et al. (2010). Plant O-hydroxyproline arabinogalactans are composed of repeating trigalactosyl subunits with short bifurcated side chains. *Journal of Biological Chemistry*, *285*, 24575–24583.

Tryfona, T., Liang, H.-C., Kotake, T., Kaneko, S., Marsh, J., Ichinose, H., et al. (2010). Carbohydrate structural analysis of wheat flour arabinogalactan protein. *Carbohydrate Research*, *345*, 2648–2656.

Tryfona, T., Liang, H.-C., Kotake, T., Tsumuraya, Y., Stephens, E., & Dupree, P. (2012). Structural characterization of Arabidopsis leaf arabinogalactan polysaccharides. *Plant Physiology*, *160*, 653–666.

Van Holst, G.-J., & Clarke, A. E. (1985). Quantitation of arabinogalactan-protein in plant extracts by single radial gel diffusion. *Analytical Biochemistry*, *148*, 446–450.

Van Holst, G.-J., & Clarke, A. E. (1986). Organ-specific arabinogalactan-proteins of *Lycopersium peruvianum* (Mill.) demonstrated by crossed electrophoresis. *Plant Physiology*, *80*, 786–789.

Woods, E. F., Lilley, G. G., & Jermyn, M. A. (1978). The self-association of glycosyl phenylazo dyes (Yariv antigens). *Australian Journal of Chemistry*, *31*, 2225–2238.

Yariv, J., Lis, H., & Katchalski, E. (1967). Precipitation of arabic acid and some seed polysaccharides by glycosyl phenylazo dyes. *Biochemical Journal*, *105*, 1c–2c.

Yariv, J., Rapport, M. M., & Graf, L. (1962). Interaction of glycosides and saccharides with antibody to the corresponding phenylazo glycosides. *Biochemical Journal*, *85*, 383.

Zou, Y. F., Zhang, B. Z., Inngjerdingen, K. T., Barsett, H., Diallo, D., Michaelsen, T. E., El-zoubair, E., & Paulsen, B. S. (2014). Polysaccharides with immunomodulating properties from the bark of Parkia biglobosa. *Carbohydrate Polymers*, *101*, 457–463.