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Note

Solid state ^{13}C-n.m.r. spectra of *Vigna* primary cell walls and their polysaccharide components

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Solid-state n.mr. spectroscopy is a promising method for determining the covalent structure of polysaccharides *in situ* in plant cell walls. By discriminating between mobile and immobile elements in the polysaccharide framework, it may help to elucidate how the loosening of primary cell walls permits and directs plant growth, and how growth is terminated when the walls become rigid. However, a study of cell-wall softening in ripened fruit[1] was not very productive due to the broad resonances and lack of detailed assignments.

Cross polarisation–magic-angle-spinning (c.p.–m.a.s.) solid-state ^{13}C-n.m.r. spectra are similar to spectra for the solution state, apart from lower resolution. However, it is unwise to assign resonances for the solid state on the basis of solution spectra, because there may be large changes in chemical shifts due to changes in conformation[2] or packing effects[3]. In particular, the different solid forms of cellulose have c.p.–m.a.s. spectra that are very different from one another[4,5] and from the ^{13}C-n.m.r. spectrum of cellulose in solution[6], due mainly to differences in conformation. Galacturonan chains also can exist in several conformations that pack in different ways[7], but it is not known whether these give different ^{13}C-n.m.r. spectra. Less is known about the other non-cellulosic polysaccharides of primary cell walls, but, as for cellulose, it is possible that the manner of their biosynthesis as parallel chains may give rise to stable but entropically unfavourable packing arrangements that cannot be reproduced by precipitation of the isolated polymers *in vitro*.

In principle, this problem can be addressed by solid-state n.m.r. spectroscopy of the intact cell walls. A complication, however, is that the c.p.–m.a.s. spectra of cell-wall materials are sensitive to moisture content and excessive milling[8], probably due to effects on the conformations of the polymers.

The contributions of various polysaccharides to the c.p.–m.a.s. ^{13}C-n.m.r. spectrum of primary cell walls from *Vigna radiata* (L.) Wilczeck hypocotyl have now been identified by successive removal of the polysaccharides from the cell walls and by obtaining spectra from the hydrated solid residue at each stage. The assumption that none of the extraction steps disturbed the conformation of the non-extracted polymers is probably valid for all but the final cellulosic residue.

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Vigna hypocotyls are a typical dicot tissue, widely used in studies of plant growth and its hormonal control[9]. Kato and Matsuda[10] found 27% of cellulose, 26% of hemicelluloses, and 47% of pectin in the cell walls, using a fractionation scheme that normally gives some cross-contamination between the fractions. They isolated a fucogalactoxyloglucan[11] (14% of the non-cellulosic polysaccharides), and the hemicelluloses also appeared to include small proportions of xylan.

The pectic fraction decreased from > 50% to just under 40% of the cell walls with increasing maturity of the hypocotyls. In the more mature tissues, just over 50% of the galacturonic acid residues in the pectic fraction were methyl-esterified and contained 40% of neutral residues, mainly (1→4)-linked β-galactopyranose and (1→5)-linked α-arabinofuranose[12-14].

The c.p.–m.a.s. ^{13}C-n.m.r. spectrum of *Vigna* primary cell walls, prepared so as to minimise physical and chemical changes, is shown in Fig. 1A. In order to emphasise that these are typical dicot cell walls, the corresponding spectrum from primary cell walls of mature stem tissue from an unrelated dicot, forage kale[15] (*Brassica oleracea L.*), is shown in Fig. 1B. Primary cell walls of monocots are more variable in composition[16].

The pectic fraction of the *Vigna* cell walls was removed by a two-stage extraction procedure which reduced the total uronic acid content from 19.8% to 8.6%. The spectrum of the residue (Fig. 1C) was depleted in the following resonances from pectic (1→4)-α-D-galacturonan: 171–175 (C-6), 96–102 (C-1), 80.3 (C-4), and 69–70 p.p.m. (C-2,3,5). These chemical shifts are similar to those in the published solution spectra[6,13], except for that of the C-1 resonance: some detail in the region 96–99 p.p.m. was removed after depectination and may be attributable to the resonance of C-1 of α-D-galactosyluronic acid residues, shifted upfield from that (100 p.p.m.) in the solution state. This difference could reflect changes in conformation due to association of the chains. Signals from the neutral pectic chains[17,18] were apparently obscured by the resonances for cellulose and hemicellulose.

Treatment of the cell walls with pectin esterase (Fig. 1D) reduced the degree of methyl-esterification from 53% to 19%, and removed the signals at 53.6 and 171 p.p.m. for COOMe of methyl α-D-galactosyluronate residues. Alkaline de-esterification (Fig. 1E), which also removed OAc substituents, eliminated the resonances at 53.6 and 171 p.p.m. together with the resonances for OAc at 21.3 and 173 p.p.m. Thus, the group of overlapping resonances for carboxyl at 170–175 p.p.m. may be assigned as follows: 171 p.p.m., methyl α-D-galactosyluronate; 173 p.p.m., OAc; 175 p.p.m., non-esterified galactosyluronic acid; as in solution [19,20]. The pH at which the walls were prepared was too high for CO_2H to contribute to the spectra. There was a small residual contribution from peptide carbonyl groups [the protein content of the cell walls (N × 6.25) was 5.0%].

An attempt to distinguish between C-6 of fucose and rhamnose by treatment with α-L-fucosidase left the resonance at 18 p.p.m. intact (spectrum not shown), but it was removed by depectination and may be assigned tentatively to rhamnose as in solution. The corresponding resonance for fucose was not located, probably due to the low signal: noise ratio.

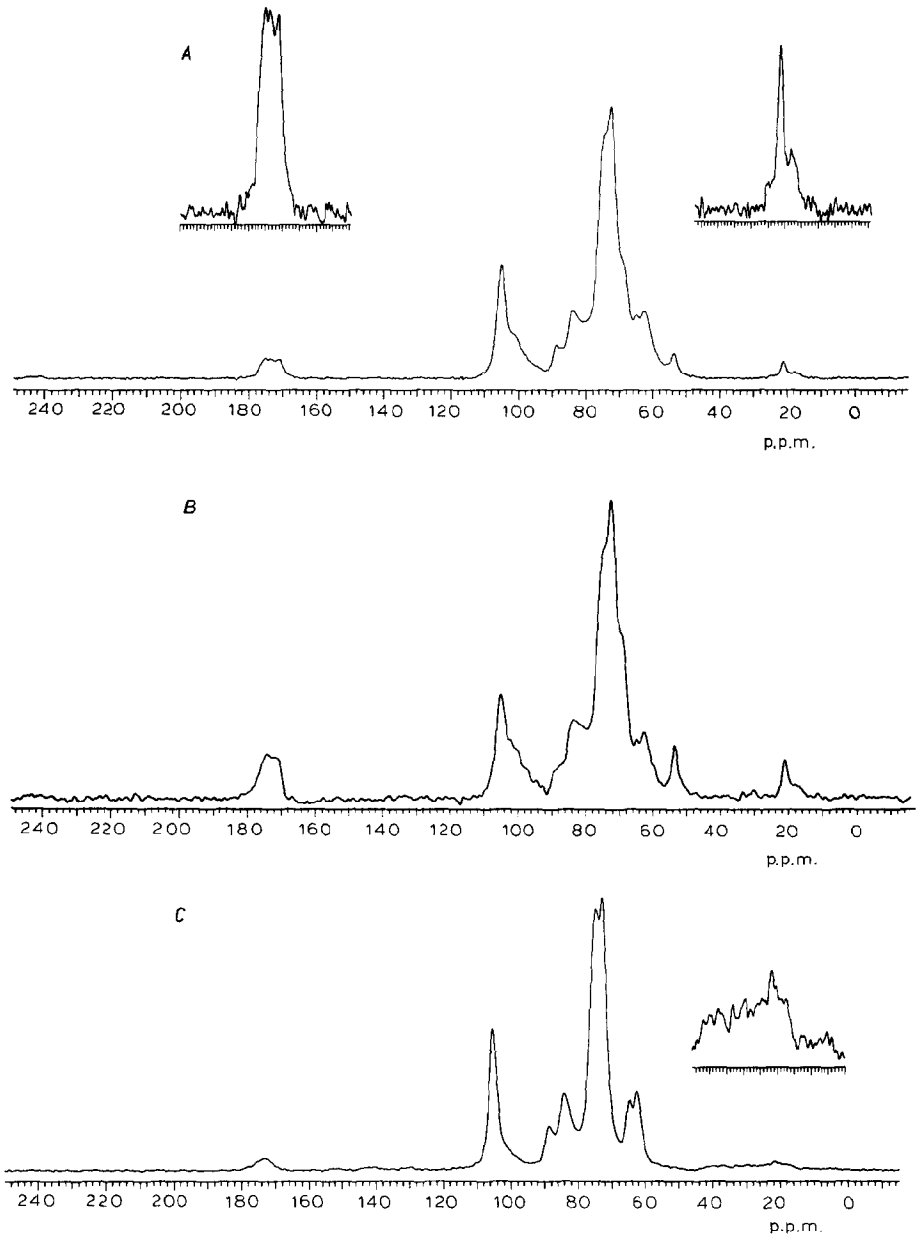
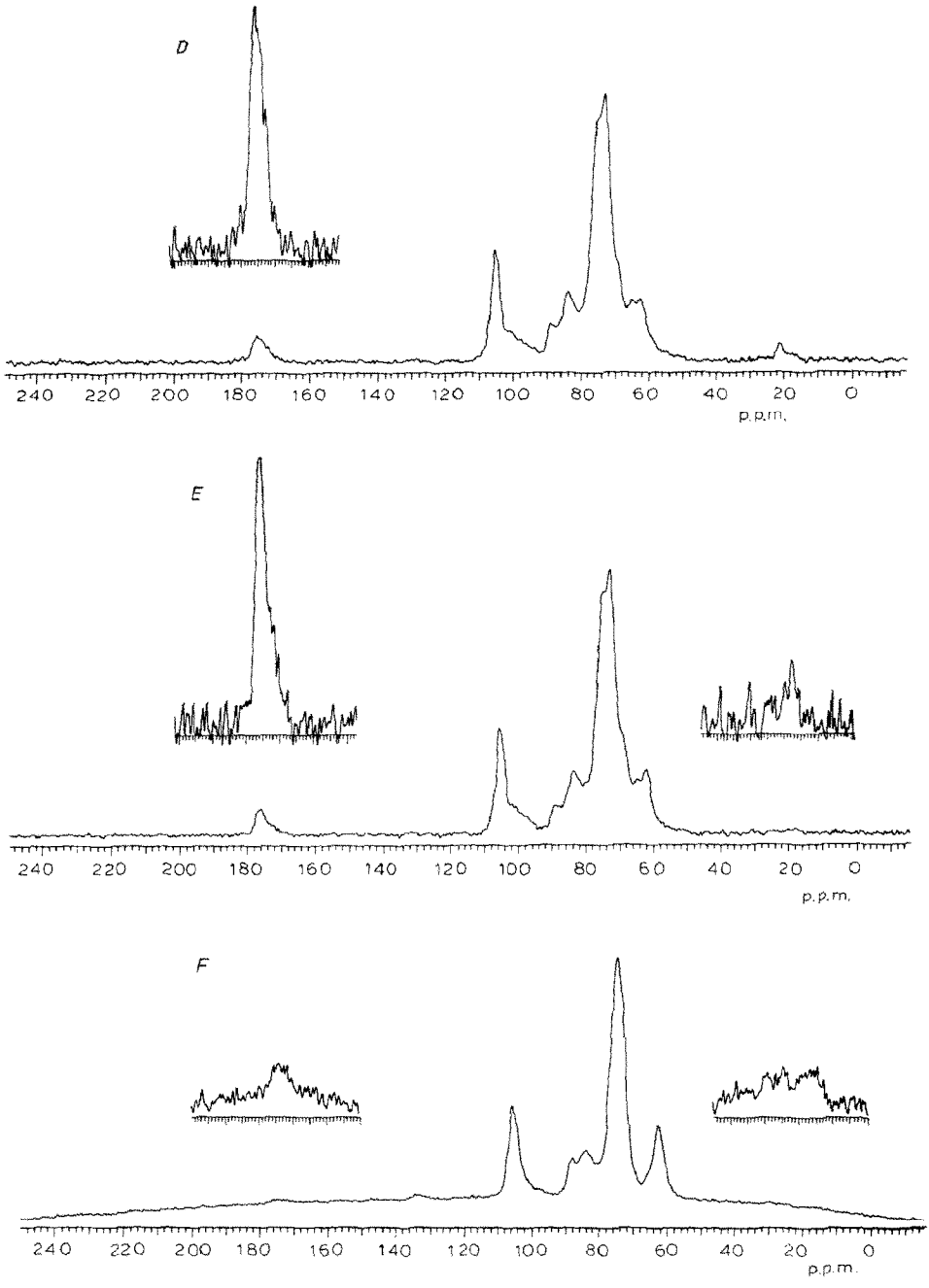


Fig. 1. C.p.–m.a.s. ^{13}C-n.m.r. spectra of primary cell walls: *A*, *Vigna* native state; *B*, *Brassica*; *C*, *Vigna* residue from pectin extraction; *D*, *Vigna* de-esterified with pectinesterase; *E*, *Vigna* de-esterified with mild alkali; *F*, *Vigna* cellulosic residue from strong alkali extraction. Inserts show x10 vertical scale expansion.


D
240 220 200 180 160 140 120 100 80 60 40 20 0
p.p.m.
E
240 220 200 180 160 140 120 100 80 60 40 20 0
p.p.m.
F
240 220 200 180 160 140 120 100 80 60 40 20 0
p.p.m.

Solution-state ^{13}C-n.m.r. data for xyloglucans have been published[6,21], although not assigned fully. A solution of the extracted *Vigna* xyloglucan in methyl sulfoxide gave a ^{13}C-n.m.r. spectrum similar to those reported (results not shown). However, extraction of the hemicellulose is one step where the assumption that the residue remains unchanged may not be valid: cellulose I is typically transformed into cellulose II, and this occurs more readily with primary than with secondary cell walls[22]. Fig 1F shows that extraction with alkali broadened the resonance at 105 p.p.m. for C-1 of its β-D-glucan and removed the resonances at 72.7 and 64.9 p.p.m. (C-6), as would be expected for the transition cellulose I → II. This broadening obscured most of the evidence for loss of xyloglucan. However, Fig. 1E suggests that the resonance for C-1 of its β-D-glucopyranose residues may be closer to (and obscured by) that for cellulose than the shift of ~102 p.p.m. found for this resonance in solution spectra.

In addition to cellulose I, there was clearly a large amount of amorphous, or crystallite-surface, cellulose present (C-4, 84.2 p.p.m.; C-6, 62.5 p.p.m.), which was not affected by extraction with alkali. The ratio of cellulose I to amorphous cellulose was much less than in cotton[4] or wood cellulose, or in the crystalline celluloses normally used in structural studies[4,5], as X-ray and electron diffraction of primary cell walls have shown[22].

It is concluded that the majority of the chemical shifts of the ^{13}C resonances from the non-cellulosic polysaccharides of primary cell walls are similar in the solid state and in solution, but that there is some evidence (tentative due to overlap of signals) of changes in chemical shift for the resonance of C-1 of galacturonans and xyloglucans. This situation may reflect the conformations of the chains *in vivo*, which are not necessarily identical with those adopted in the solid state *in vitro*. The large proportion of amorphous cellulose probably dominates the interaction of the microfibrils with non-cellulosic polymers bound to their surface.

EXPERIMENTAL

Preparation of cell walls — *Vigna radiata* hypocotyls (150 g) with the apical hook removed were homogenised in (*1*) 1:1 $CHCl_3$–MeOH (600 mL), (*2*) 10 mM NaOAc–3 mM KCl–2mM $MgCl_2$–mM $CaCl_2$ (pH 6.0, 600 mL), (*3*) Triton X-100 (600 mL, 2 $g.L^{-1}$) with the same buffer composition as in (*2*). The cation composition of the buffer was chosen to match the approximate cation concentrations in contact with *Vigna* cell walls *in vivo*[23]. The cell walls were washed extensively with buffer and water, and excess of liquid was removed by suction. The cell walls were in contact with ice throughout their preparation, and were not fully air-dried. Non-lignified cell walls from *Brassica oleracea* were prepared as described previously[15].

Selective extraction. — Pectic polysaccharides were solubilised by β-elimination in 50mM NaOAc buffer (pH 5.5) for 20 min at 120° followed by extraction with 50mM cyclohexane-1,2-diamine tetra-acetic acid–0.4M KOH–aqueous $NaBH_4$ (1 $g.L^{-1}$) and extensive washing with water[16]. Hemicelluloses were extracted from the residue with 4M

KOH. Uronic acid was determined colorimetrically in extracts after partial acid hydrolysis[16].

Enzymic degradation. — *Vigna* cell walls (290 mg, dry matter) were suspended in phosphate buffer (20 mL, 0.1M in Na^+, pH 7.5), incubated for 16 h at 16° with 650 U of purified tomato pectinesterase (EC 3.1.1.11, Sigma), then washed with the same buffer, water, and the Na/K/Mg/Ca buffer used above. The degree of esterification of the pectic fraction was determined by titration of the cell walls[14]. The tomato tissue from which the pectinesterase is commercially prepared also contains polygalacturonases and a (1→4)-β-D-galactanase[24,25]. When contaminating activities of these enzymes were present, 80 U of pectinesterase were incubated with 1 mg of citrus galacturonan (Sigma) or *Allium* (1→4)-β-D-galactan[26] for 3 h at 25° in 1 mL of buffer, and the hydrolytic activity was measured on the basis of the release of reducing groups. Traces of activity against both substrates were detected in a buffer suitable for this purpose[24], namely, 50mM Na acetate–150 mM NaCl (pH 4.5), but no activity was found in the phosphate buffer (pH 7.5) used for enzymic de-esterification of the cell walls. Therefore, it is unlikely that contaminants in the pectinesterase preparation depolymerised any of the cell-wall polysaccharides. Similar conditions were used for incubation with 0.5 U of purified bovine-liver α-L-fucosidase (EC 3.2.1.51, Sigma).

Alkaline de-esterification. — *Vigna* cell walls (315 mg, dry matter) were stirred in 0.25M NaOH–aqueous 75% MeOH for 16 h at 16° and washed with the Na/K/Mg/Ca buffer and water. There was probably a small amount of galacturonan depolymerisation under these conditions, but the MeOH would prevent any solubilisation.

^{13}C-N.m.r. spectroscopy. — Natural-abundance c.p.–m.a.s. ^{13}C-n.m.r. spectra were recorded at ambient temperature on a Varian VXR-300 spectrometer operating at 75.43 MHz (contact time was 1 ms; pulse width, 90°; acquisition time, 19.2 ms; and relaxation delay, 1 s). The number of transients varied from 1000 to 5400. A spin rate of 3.3–5.3 kHz in a Kel-F rotor gave adequate suppression of spinning side-bands, as shown by the acquisition of an essentially identical spectrum for *Brassica* walls, using the pulse sequence described by Dixon *et al.*[27] for total side-band suppression, and by the absence of spectral changes following alterations in spin rate.

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