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Use of Chemical Fractionation and Proton Nuclear Magnetic Resonance to Probe the Physical Structure of the Primary Plant Cell Wall[1]

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ABSTRACT

Proton magnetic resonance has been used to monitor the microscopic physical properties of etiolated hypocotyl cell walls from *Phaseolus vulgaris* L. at all stages in a series of chemical fractionations with ammonium oxalate and potassium hydroxide. Solid echo measurements indicate that 75% of the polymers in the intact cell wall, including the cellulose and most of the hemicelluloses, are arranged such that there is almost complete restraint of molecular motion. The chemical fractionations generally altered the physical structures of the remaining cell wall components. Digestion with 0.25% ammonium oxalate/oxalic acid solubilized the pectin and increased the mobility of the hemicellulose I component. Extraction with 4% potassium hydroxide removed the hemicellulose I component and loosened the hemicellulose II. Further extraction with 24% potassium hydroxide removed the hemicellulose II and loosened some of the cellulose. The cellulose crystallinity, as monitored by Jeener echo measurements decreased from 83% to 63% during these fractionations. We conclude that, while hemicellulose I is firmly attached to hemicellulose II, it is not in a closely packed structure. Hemicellulose II is strongly bound to cellulose and has a much more closely packed structure.

There has been some recent acceleration in progress toward a quantitative architectural model of the plant cell wall (4, 11, 14, 16, 22). The substantial literature on the chemical structures of the constituents (for reviews see 2, 5, 15), on the physical form of the polysaccharides (6, 7, 19), and on the role of cell walls in growth control (3, 4, 18) provides the foundations from which it may be possible to describe physical and chemical relationships between polymers as they exist in the functioning cell wall.

Broadline proton nuclear magnetic resonance (^{1}H-NMR) has been used to study microscopic physical properties of the constituent polymers both separately and as they occur in whole cell wall preparations from etiolated hypocotyls of *Phaseolus vulgaris* (12, 14, 20). Further characterization, especially of the interactions between the constituent polymers, can be achieved if the properties of wall constituents in native walls can be related to those observed after modification using chemical fractionation procedures.

We have used the ^{1}H-NMR methods reported earlier (12–14, 20) to describe the properties of *P. vulgaris* etiolated hypocotyl cell wall fragments before and after each stage of the commonly used chemical fractionation with ammonium oxalate and KOH (10). The aims of this work were to discover whether the physical properties of the remaining materials were affected by the chemical fractionation of the cell walls and to assess the implications of fractionation studies for models of *in vivo* cell wall structure.

MATERIALS AND METHODS

All water was deionized and glass distilled. Deuterium oxide (^2H_2O) (greater than 99.9% pure) was obtained from Merck Sharp and Dohme, Montreal, Canada.

Plant Materials

Bean seeds (*Phaseolus vulgaris* L. cv Top Crop Green Pod) were obtained locally and were germinated in moistened vermiculite at 23°C in the dark. Seedlings were harvested when the lengths of the hypocotyls were 12 to 15 cm (7–8 d).

Cell Wall Preparation

The hypocotyl segments (the first 2 cm below the cotyledon insertion) were frozen to −40°C, homogenized to a powder in a Sorvall Omnimixer, and disrupted twice in an Edebo X-press (Biotec Inc, Rockville, MD). The thawed homogenate was dispersed by sonic disruption, washed with water until no cytoplasmic contamination was visible under dark field light microscopy, and lyophilized.

Cell Wall Fractionation

The wall material was digested with 30 mL (approximately 5 mg/mL) ammonium oxalate/oxalic acid (0.25% w/v of each) five times for 1 h at 80°C (to remove most pectic polysaccharide). The residual walls were washed with deionized water to neutral pH and lyophilized. Hemicellulose I (10)

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[3] Abbreviations: $M_{2interpair}$, interpair second moment; T_{1D}, dipolar relaxation time.

was removed by digestion with 30 mL (2 mg/mL) 4% (w/v) KOH twice for 24 h at room temperature under nitrogen. The residue was washed with deionized water to neutral pH, and lyophilized. Further alkaline extraction to remove hemicellulose II (10) was with 10 mL (2 mg/mL) 24% KOH for 20 h at 5°C under nitrogen. The residual wall material (cellulosic fraction) was recovered by centrifugation, washed with water to neutral pH, and lyophilized.

Before and after each extraction, weighed samples were removed, washed three times with ^2H_2O to remove exchangeable protons, and lyophilized. The exchanged sample was packed into a 4 mm i.d. NMR sample tube, rehydrated with enough ^2H_2O to wet the sample with a small (1 mm) layer above the material in the tube, and stored frozen for NMR study.

NMR Methods

All of the ^{1}H-NMR measurements were taken at 24°C on a modified Bruker SXP 4-100 pulse NMR spectrometer operating at 90 MHz. Data were acquired on a Nicolet Digital Oscilloscope. The supervision of the experiment was performed by a MicroVAX 1 microcomputer which was also used for data analysis. A locally built pulse programmer was used to control the experiments (17). Details of the relevant NMR concepts and the experimental pulse sequences were described by MacKay *et al.* (14).

RESULTS

Chemical Results

The sugar compositions of the residual cell walls after each extraction stage were similar (Table I) to those reported in other studies on *P. vulgaris* hypocotyl cell wall preparations (21). The chemical composition of the material solubilized during each extraction was not determined.

The relative numbers of protons of cellulose, hemicelluloses I and II, and pectin in each of the preparations are in Table I. Because our ^{1}H NMR signals scale with numbers of protons, we have taken into account the slightly different proton densities of the constituents (14).

NMR Results

Solid echo and Jeener echo measurements were performed on each of the four sample preparations: whole cell walls, the residual after digestion with 0.25% ammonium oxalate/oxalic acid, the residual after further digestion with 4% KOH, and the final residual after digestion with 24% KOH.

Table I. *Polymer Composition of Bean Cell Walls Determined by Gravimetric Analysis and Proton Density*

Polymer	Gravimetric Composition	Proton Density
	%	
Pectin	18	21
Hemicellulose I	24	18
Hemicellulose II	15	15
'Cellulose'	43	46

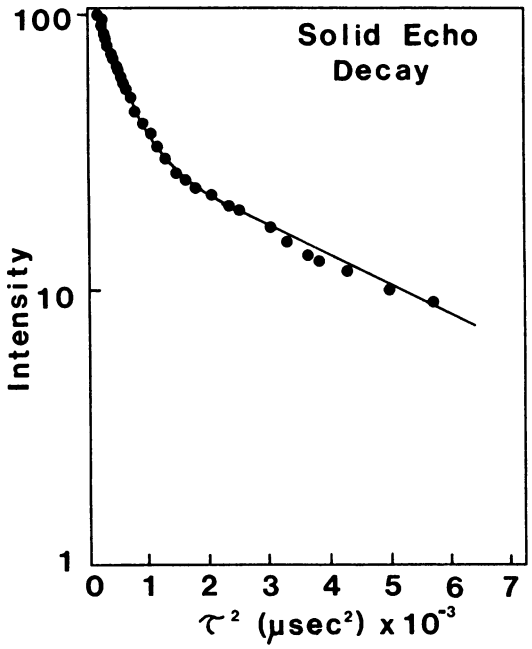

Figure 1. Solid echo decay curve for cell wall-minus pectin preparation from etiolated bean hypocotyls.

The peak amplitudes of the solid echos were measured as a function of the time, t, between the two 90° pulses ($90^\circ_x - t - 90^\circ_y$ – echo). For a homogeneous proton population, the echo peaks, $E_s(t)$, should follow the relation (ref. 1):

$$E_s(t) = E_s(0)\cdot\exp(-\tfrac{1}{2}\cdot M_{2\text{interpair}}\, t^2) \quad (1)$$

A representative solid echo decay curve for the cell wall minus pectin sample is shown in Figure 1. Although this curve cannot be fitted by equation 1 alone, it was relatively well fitted by the sum of two exponential components when a nonlinear chi-squared minimization program (8) was used. Table II lists the $M_{2\text{interpair}}$ values and the relative proportions for the four samples. All samples could be fitted by only two components, one with an $M_{2\text{interpair}}$ value of 4×10^9 s^{-2} and the other with $M_{2\text{interpair}}$ equal to 4×10^8 s^{-2}.

Peak amplitudes of the Jeener echos ($90^\circ_x - \tau_1 - 45^\circ_y - \tau_2 - 45^\circ_y$ – echo) were measured as a function of the time between the two 45° pulses, τ_2. For a homogeneous proton population, the Jeener echo decay curve should follow a single exponential:

$$E_j(\tau_2) = E_j(0)\cdot\exp(-\tau_2/T_{1D}) \quad (2)$$

A representative Jeener echo decay curve for the cell wall minus pectin sample is plotted in Figure 2. For all four samples the Jeener echo decay curves were best fitted by the sum of two exponential components. The relative proportions and T_{1D} values are listed in Table III. The errors are larger than those for the solid echo measurements because the two dipolar relaxation times differed by only a factor of 3.

Our experience has been that, although there is some variation in polymer composition between different cell wall

Table II. *$M_{2interpair}$ Measurements on Bean Cell Walls and Cell Wall Residues*

Estimated standard errors in the $M_{2interpair}$ values and their proportions are less than 5%.

Preparation	$M_{2interpair}$	
	$4.4 \times 10^9 \cdot s^{-2}$	$4 \times 10^8 \cdot s^{-2}$
	% of total signal	
Whole wall	75.4	24.6
Wall minus pectin	74.0	26.0
Wall minus pectin minus hemicellulose I	78.5	21.5
Wall minus pectin minus hemicellulose I minus hemicellulose II	77.7	22.3

preparations, the physical properties observed by proton NMR are similar for each constituent fraction.

DISCUSSION

The chemical heterogeneity of the plant cell wall has hindered understanding of *in vivo* relationships within the complex. Much information has been derived from preparations which have been fractionated with ammonium oxalate and KOH (10). An implicit assumption has been that damage to the chemical structure of constituents is relatively minor. Unambiguous testing for degradation during fractionation treatments is not trivial. In one instance, solubilization using the organic base *N*-methylmorpholine oxide has been reported to hydrolyse β-1,4-D-glucan chains from cellulose (9). In this paper we show that ammonium oxalate and KOH fractionation treatments modify the physical structure of remaining constituents.

Physical Measurements

We employ two types of solid state NMR measurements to characterize the physical structure of the wall preparations: the solid echo ($M_{2interpair}$) and the Jeener echo (T_{1D}). Both of these echos arise exclusively from protons on molecules that are either solid or undergo anisotropic motion on the ^1H NMR timescale of 10^{-5} s. This time scale is determined by the strength of the proton-proton dipolar interactions. For the hydrated cell wall preparations used here, this means that we observe signals from only the polymeric components of the cell walls (14).

For all four samples, the solid echo measurements distinguished between two components with different $M_{2interpair}$ values. One component had an $M_{2interpair}$ of about 4×10^9 s^{-2} and the other had an $M_{2interpair}$ value an order of magnitude smaller. The former arises from polysaccharide molecules that are practically rigid and the latter from molecules undergoing much more motion. By much more motion, we mean that on the 10^{-5} s timescale the molecules undergo relatively large anisotropic excursions. The assignment of these components has been discussed previously (14).

Jeener echo studies on preparations of cellulose (13) and cell walls (14) have been interpreted in terms of contributions from paracrystalline and crystalline components. It has been demonstrated (13) that crystalline cellulose has a T_{1D} value of 30 to 60 ms and a second moment corresponding to the rigid lattice M_2 value, and paracrystalline cellulose has a T_{1D} value of 10 ms or less and an M_2 value that is about 70% of the rigid lattice value. In cell wall preparations (14), we have assigned the T_{1D} component with the longer T_{1D} to the crystalline cellulose, and that with the shorter T_{1D} to paracrystalline cellulose and other polysaccharides. We point out that the proportions of the crystalline components reported here should be interpreted as lower limits due to the possibility of spin diffusion between the two components (13, 14). The Jeener echo measurements are less accurate than the solid echo measurements since the two T_{1D} components differed by only a factor of 3 (Table III).

Whole Cell Wall

Solid echo measurements indicate that the intact wall has a restricted fraction of 75%. Our interpretation is that this corresponds to cellulose, hemicellulose II and perhaps some hemicellulose I. The more mobile fraction would then correspond to pectin which possesses 21% of the protons in the sample. The Jeener echo measurements indicate the presence of a crystalline cellulose component of 38%. Because the total cellulose component is 46% (Table I), this suggests that the cellulose was about 80% crystalline.

Wall Minus Pectin

If the sole action of the ammonium oxalate/oxalic acid digestion procedure were to remove the pectin without affect-

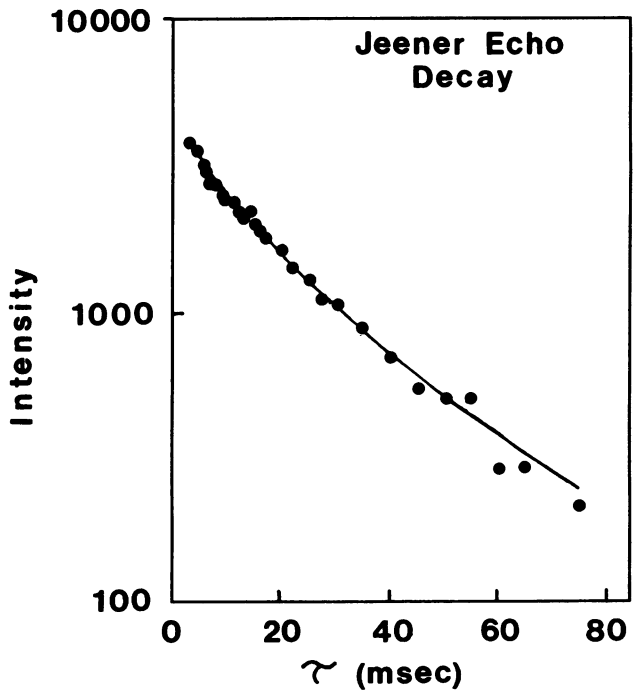

Figure 2. Jeener echo decay curve for cell walll-minus pectin preparation from etiolated bean hypocotyls.

Table III. *T_{1D} Measurements on Bean Cell Walls and Cell Wall Residues*

Estimated standard errors in the T_{1D} values are of the order of 15–30%.

Preparation	T_{1D}	
	% of signal	msec
Whole wall	38	30.9
	62	9.8
Wall minus pectin	45	35.7
	55	12.4
Wall minus pectin minus hemicellulose I	49	58.0
	51	17.0
Wall minus pectin minus hemicellulose I minus hemicellulose II	61	25.0
	39	3.5

ing the remaining polysaccharide, one would expect the proportion of the sample in the restricted fraction to increase considerably. In fact, the restricted fraction remained almost the same at 74%. The most likely interpretation is that most of the hemicellulose I component has been loosened by the digestion. The crystallinity of the cellulose as measured by the Jeener echo experiments was still about 80% (*i.e.* 45% of the preparation which was 48% cellulose), so it appears that the cellulose microfibrils are minimally affected by the digestion. We conclude, therefore, that there is some change in the physical structure of the residual sample upon ammonium oxalate/oxalic acid digestion but this change is probably limited to the hemicellulose I component, all of which is much more mobile than when it is in the intact cell wall.

Wall Minus Pectin Minus Hemicellulose I

Digestion by 4% KOH is said to extract the hemicellulose I fraction, leaving hemicellulose II and cellulose intact (10). If this were strictly true, we would expect the entire sample to undergo only restricted motion. The solid echo measurements revealed a restricted fraction of 79% indicating that most (84%) of the hemicellulose II had been loosened by the 4% KOH digestion. The cellulose itself was not affected so much; its crystallinity was reduced from 80 to 65%.

Wall Minus Pectin Minus Hemicelluloses I and II

After digestion by 24% KOH, the residual sample is largely made up of cellulose alone. The solid echo measurements indicate that only 78% of this cellulose is in the restricted fraction while 22% has been loosened significantly. The crystallinity of the cellulose was found to be about 60%.

Implications for Cell Wall Structure

Our proton NMR experiments on samples before and after chemical fractionation provide some insight into the prevailing order and motion within a particular fraction before and after treatment. The properties before treatment reflect the behavior of constituents either without disruption (whole wall) or after removal of one constituent and a nonsolubilizing treatment of remaining components. The ammonium oxalate/oxalic acid treatment represents heating at a weakly acid pH. Clearly, much of the pectic material is extracted and the hemicellulose I which will be solubilized by heating with 4% KOH is altered to allow more molecular motion. It also clear that the extraction with 4% KOH removes the hemicellulose I and alters behavior of the protons in the hemicellulose II. We interpret these results as indicating that in the whole wall, the hemicelluloses and cellulose are in relatively close proximity to each other and arranged and ordered in such a way that there is substantial constraint of molecular motion. This is particularly notable in the observation that the restricted fraction makes up 75% of the whole wall and approximately 40% is in a crystalline state. The increased motions in the hemicellulose I after oxalate treatment implies that hemicellulose I is relatively easily 'loosened' under mildly acidic conditions. We conclude that, while this fraction was firmly attached to the inner hemicellulose II, it was not in a closely packed structure and thus was susceptible to the dilute acid treatment. The 4% KOH treatment had a parallel effect on the hemicellulose II, but the apparent requirement for more extreme pH implies not only strong binding with cellulose but that the hemicellulose II is in a more closely packed structure than is hemicellulose I. This rationalization is consistent with the widely held rationale for the extraction protocol.

In conclusion, it is clear that, while our NMR experiments provide some insight into the prevailing order and motion within plant cell walls before chemical treatment, they also indicate that the extraction protocols which we have used have major influences on noncellulosic components before they are solubilized.

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