Solid-State ^{13}C NMR Investigation of Molecular Ordering in the Cellulose of Apple Cell Walls

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A resolution-enhanced ^{13}C nuclear magnetic resonance (NMR) spectrum of cell wall material isolated from Braeburn apples has revealed a pair of signals assigned to cellulose chains exposed on crystal surfaces, providing evidence for a high degree of molecular ordering on those surfaces. Proton spin relaxation editing revealed no detectable amorphous cellulose and provided an estimate of about 23 chains in a typical crystallite. Cellulose in apple cell walls was found to be distinctive, with triclinic Iα and monoclinic Iβ crystalline forms present in similar proportions. Hence, its composition is intermediate between the family of bacterial and *Valonia* celluloses, rich in the triclinic crystalline form, and the family of cotton and ramie celluloses, in which the monoclinic form is dominant. Implications for apple crispness are discussed.

Keywords: *Cellulose in apple cell walls*

INTRODUCTION

Plant cell walls have a range of functions that include cell shape, control of cell growth, disease resistance, and mechanical strength (Fry, 1988). Mechanical strength can be expected to influence food texture and digestibility. Cellulose is a component of the cell walls of all dicots and monocots (Bacic et al., 1988). Cellulose microfibrils cross-linked by xyloglucan molecules are thought to provide the framework about which other polymeric networks are arranged to form dicot cell walls (McCann and Roberts, 1991; Carpita and Gibeaut, 1993).

Little is known about the nature of molecular ordering of cellulose in the primary cell walls of plants. Frey-Wyssling (1954) reported "astonishingly poor" crystallinity in cellulose prepared from the growing root tips and coleoptiles of corn. Solid-state NMR spectroscopy has been used to study plant material taken from leaves and stems (Himmelsbach et al., 1983; Maciel et al., 1985; Jarvis, 1990; Jarvis and Apperley, 1990; McBride, 1991), potato tubers (Garbow et al., 1989), and apple fruit (Irwin et al., 1984, 1985, 1992) and seeds (O'Donnell, 1981; Haw and Maciel, 1983; Jarvis and Apperley, 1990). However, the peaks in the published spectra are too broad for clear distinction between crystalline forms of cellulose.

Native celluloses are believed to be composites of two crystalline forms, designated Iα and Iβ (Atalla and VanderHart, 1984; VanderHart and Atalla, 1984). The Iα form is triclinic, the Iβ form monoclinic (Sugiyama et al., 1991). These forms can be distinguished by ^{13}C nuclear magnetic resonance (NMR) spectra obtained with cross polarization (CP) and magic-angle spinning (MAS) (Atalla and VanderHart, 1984), by infrared spectroscopy (Debzi et al., 1991), or by electron diffraction (Sugiyama et al., 1991).

CP/MAS NMR spectra of pure celluloses from various sources have been improved by digital resolution enhancement (Cael et al., 1985; Belton et al., 1989; Debzi et al., 1991), but this technique is not as easily applied to spectra of plant cell walls because it results in deterioration of a signal-to-noise ratio which is already depressed by dilution of the cellulose by other cell components, e.g., hemicelluloses and pectin. Signal-to-noise ratios can, however, be improved by lengthy data averaging. This paper reports results from such an experiment, along with results from a proton spin relaxation editing experiment designed to separate subspectra of crystalline and non-crystalline material within cell walls.

Apple cell walls were chosen as a model system to work with because the chemical composition of their polymeric components has been thoroughly investigated (Stevens and Selvendran, 1984) and, furthermore, solid-state NMR has previously been successful in examining the shape and flexibility of the pectic polymers in apple cell walls (Irwin et al., 1984, 1985, 1992).

EXPERIMENTAL PROCEDURES

Preparation of Apple Cell Walls. Apples (*Malus domestica* Borkh. cv. Braeburn) were grown in a Central Otago orchard, New Zealand, and stored for 2 months at 0.5 °C and then for 3 months at 4 °C. The ripe apples were then quartered, the core region was discarded, and the remainder was immediately frozen in liquid nitrogen. The frozen apple was ground under liquid nitrogen. The frozen apple powder (75 g) was mixed with phenol saturated with 0.5 M Tris buffer (150 mL, pH 7.5) to inactivate enzymes (Huber, 1992). The mixture was homogenized using an Ultra-Turrax. The resulting slurry was centrifuged at 5000*g* for 20 min; the supernatant was removed, and the cell walls were washed with 80% ethanol (150 mL). The cell walls were centrifuged at 5000*g* for 20 min, and the supernatant was removed. The cell walls were washed three more times with 80% ethanol (150 mL), the supernatant being discarded after each wash. The cell walls were then stored in 80% ethanol (125 mL) until required.

NMR Spectroscopy. The stored sample was air-dried overnight at 20 °C, moistened to 41% by weight with water, packed in a 7 mm diameter cylindrical sapphire rotor, and retained with Vespel end caps. A preliminary experiment showed that moisture was essential for good resolution of signals from cellulose crystal surfaces. Earlier studies of wood have also shown that moisture enhances differences in molecular mobility between cellulose and noncellulosic components, facilitating proton spin relaxation editing (Newman, 1992). The rotor was spun at 4 kHz in a Doty Scientific magic-angle spinning probe for ^{13}C CP/MAS NMR spectroscopy at 50.3 MHz on a Varian XL-200 spectrometer.

A resolution-enhanced spectrum was generated from transient signals averaged over a period of 110 h. Each 7-μs proton

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preparation pulse was followed by a 1-ms cross-polarization contact time, 30 ms of data acquisition, and a recovery delay of 1 s before the sequence was repeated. Preliminary experiments showed that the proton spin–lattice relaxation time constant was 0.28 s, so the delay was adequate for recovery of proton magnetization.

The decoupler field strength was increased to provide a proton rotating-frame precession frequency of 70 kHz for data acquisition. Preliminary experiments showed that values greater than 60 kHz were a prerequisite for success in resolving peaks in samples similar to that discussed here. Resolution-enhanced spectra were obtained by convoluting NMR free induction decays with a function of the form

$$f(t) = \exp\{(t/T_X)^x/x - (t/T_Y)^y/y\} \quad (1)$$

If $x = 1$ and $y = 2$, this function corresponds to the Lorentzian-to-Gaussian transformation, originally designed for use in solution NMR spectroscopy (Ferrige and Lindon, 1978). The first term within the brackets causes the function to increase, counteracting exponential decay curves associated with line broadening in solution NMR. The second term prevents the weighting function from increasing indefinitely. This function has been used in solid-state NMR studies of cellulose (Cael et al., 1985; Belton et al., 1989; Debzi et al., 1991) but without theoretical justification since the decay curves associated with line broadening in solid-state NMR are generally better described by Gaussian functions. Incrementing the exponent in the first term to $x = 2$ generates a function that counteracts Gaussian line broadening. We found small improvements in resolution-enhanced spectra of microcrystalline cellulose when we used $x = 2$ and $y = 3$ instead of $x = 1$ and $y = 2$, so these values were used in the present work. We chose $T_X = 6$ ms and $T_Y = 9$ ms by trial and error.

Proton spin relaxation edited (PSRE) NMR subspectra were generated from a pair of "delayed contact" spectra labeled $S(0)$ and $S(4)$ which were obtained with proton magnetization spin locked during a delay of 0 or 4 ms between the proton preparation pulse and the CP contact time. A total of 41 728 transient signals were averaged for each value of the spin-locking delay. The principles behind PSRE NMR have been described elsewhere (Newman and Hemmingson, 1990; Newman, 1992). Briefly, coefficients of linear combinations of $S(0)$ and $S(4)$ were adjusted by trial and error to maximize mutual discrimination of signals without allowing any signal to become inverted. The selected combinations were

$$S(a) = -1.28S(0) + 3.44S(4)$$

$$S(b) = 2.28S(0) - 3.44S(4)$$

It should be noted that the sum of these subspectra is simply $S(0)$, i.e., the normal CP/MAS NMR spectrum. The coefficients given above correspond to selection of subspectra from domains with proton rotating-frame relaxation time constants of (a) 9.7 and (b) 4.0 ms, as calculated from relationships published by Newman and Hemmingson (1990) and Newman (1992).

A sample of commercial microcrystalline cellulose (Avicel) was purchased from Merck Chemical Co. The Avicel was moistened to 41% (by weight) water, and a resolution-enhanced NMR spectrum was obtained as for apple cell walls, except that the recovery delay was extended to 4 s and data averaging was limited to 44 h.

RESULTS AND DISCUSSION

The PSRE subspectra separated in Figure 2 show a high degree of discrimination between signals that can be assigned to cellulose (Figure 2a) and signals that can be assigned to pectins and hemicelluloses (Figure 2b). Assignments were based on those discussed by other authors, as follows. The signal associated with C-1 of cellulose is expected to appear at a chemical shift of 105 ppm regardless of whether the cellulose is in a crystalline or amorphous form (Atalla et al., 1980). A prominent signal is present at this chemical shift in Figure 2a, but there is

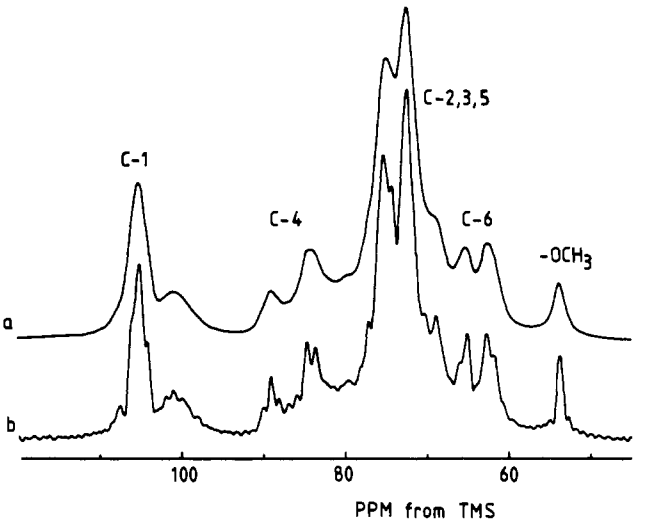

Figure 1. CP/MAS NMR spectra of Braeburn apple cell walls (a) before and (b) after resolution enhancement. The signal labeled $-OCH_3$ is assigned to pectin. All other labels refer to cellulose.

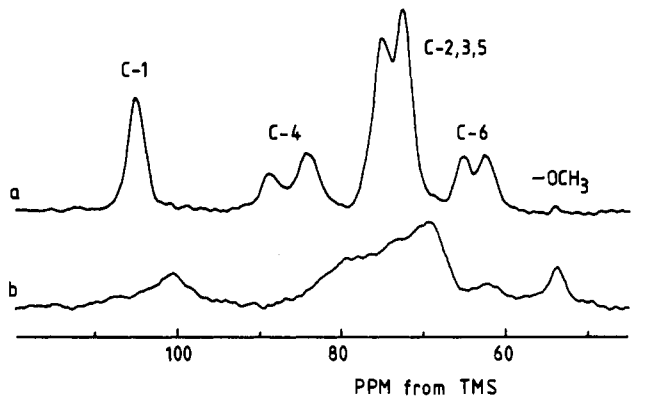

Figure 2. Proton spin relaxation edited subspectra separated from the CP/MAS NMR spectrum of Braeburn apple cell walls, showing signals assigned to (a) cellulose and (b) noncellulosic material in domains associated with proton rotating-frame relaxation time constants of (a) 9.7 and (b) 4.0 ms.

no evidence for any such signal in Figure 2b. On the other hand, signals from C-1 in the pectic fraction are expected to appear in a band from 96 to 102 ppm (Jarvis, 1990), while signals from C-1 in hemicelluloses are expected to appear in a band from 100 to 104 ppm (Maciel et al., 1985; Jarvis and Apperley, 1990). A broad band spans this range in Figure 2b, reaching a maximum at about 101 ppm, but there is no evidence for such a band in Figure 2a. We conclude that the cellulose is confined to domains in which proton rotating-frame spin relaxation is relatively slow (Figure 2a), while pectins and hemicelluloses are confined to domains in which proton rotating-frame spin relaxation is relatively rapid (Figure 2b).

The presence of pectins is confirmed by a peak at 54 ppm in Figure 1, assigned to methoxyl carbon in methyl α-D-galactosyluronate residues of pectins (Jarvis, 1990). Proton spin relaxation editing associated this peak with others assigned to pectins and hemicelluloses in Figure 2b.

Cellulose accounted for about half of the total signal response but did not necessarily account for half of the dry weight of the cell walls since some of the noncellulosic molecules might have been too mobile for efficient cross-polarization processes.

Crystallite Dimensions. It has been suggested by Earl and VanderHart (1981) that signals assigned to C-4 in

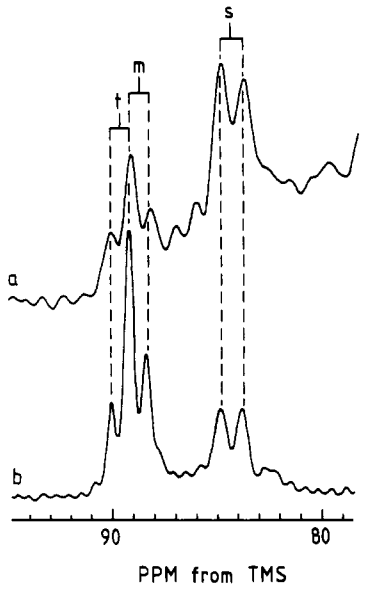

Figure 3. Portions of resolution-enhanced CP/MAS NMR spectra of (a) Braeburn apple cell walls and (b) Avicel microcrystalline cellulose. Signals assigned to C-4 in cellulose are labeled t and m for triclinic and monoclinic crystalline forms, respectively, and s for chains exposed on crystal surfaces.

cellulose can be grouped into a band near 90 ppm, associated with chains contained within crystals, and a band near 86 ppm, associated with chains exposed on crystal surfaces. The chemical shift difference between these two bands is attributed to a difference in patterns of hydrogen bonding. Each crystal-interior chain unit is hydrogen-bonded to two adjacent chains, while each crystal-surface unit is hydrogen-bonded to just one adjacent chain (Gardner and Blackwell, 1974). Earl and VanderHart (1981) studied celluloses from diverse sources and showed that the differences in relative strengths of the bands were consistent with differences in crystal dimensions estimated from diffraction studies. However, Horii et al. (1984) suggested that the band at the lower chemical shift should be assigned primarily or exclusively to disordered cellulose. The resolution-enhanced spectrum in Figure 1b has revealed fine structure not reported in earlier descriptions of this band. The details can be seen more clearly in Figure 3. Pairs of signals were resolved at 84.0 and 84.9 ppm and labeled "s" in spectra of apple cell walls (Figure 3a) and microcrystalline cellulose (Figure 3b). This fine structure is not consistent with amorphous or even partly ordered material, but it is consistent with a pair of crystallographically nonequivalent structural units on well-ordered surfaces of crystals.

The signals at 84.0 and 84.9 ppm cannot be assigned to pectins or hemicelluloses. The proton spin relaxation edited subspectrum assigned to those noncellulosic substances (Figure 2b) shows only a featureless curve over the chemical shift range 80–90 ppm, with no potential for resolution of fine detail.

Relative areas of the C-4 signals in Figure 2a were estimated by drawing vertical boundaries at 93, 87, and 79 ppm and measuring the areas enclosed. This procedure provided an estimate of 38% of cellulose chains contained in crystal interiors. A crystallite constructed from 23 chains arranged as in Figure 4 would have 39% crystal-interior cellulose, i.e., a proportion very close to that observed. This crystallite would have dimensions of 3.0 × 2.7 nm.

Ben-Arie et al. (1979) published photos of the cell walls of Golden Delicious apples showing fibrillar elements a few nanometers wide but did not attempt to place a value on the dimensions. Fibrillar elements with similar dimensions have, however, been observed in primary cell walls obtained from other sources. Chanzy et al. (1979) used both X-ray and electron diffraction to estimate widths of between 2 and 3 nm for crystallites in rose cells cultured *in vitro*. Roland et al. (1975) used electron microscopy to study cell wall material from actively elongating bean, pea, and celery cells and found fibrillar elements with diameters in the range 2.0–2.5 nm. Ruben and Bokelman (1987) used electron microscopy to detect fibrillar elements just large enough to contain nine cellulose chains in tobacco lower epidermal peels.

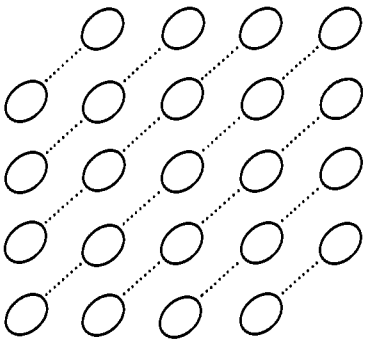

Figure 4. Representation of a cross section through a cellulose crystallite. Long axes of the cellulose chains are oriented normal to the plane of the paper. Each ellipse represents a glucose unit, and dotted lines represent hydrogen bonds.

Microfibrils. The time scale required for nuclear spin information to diffuse over a distance r is of the order of r^2/D (Abragam, 1961). The spin diffusion constant D is roughly 10^{-15} m^2 s^{-1} for proton spins in cellulose (Newman, 1992), so a time scale of the order of 6 ms would be adequate for diffusion over the crystallite dimensions suggested above. If each cellulose crystallite was surrounded by noncellulosic material, then spin diffusion would blur the distinction between observable values of spin relaxation time constants. The observation of distinct proton rotating-frame relaxation time constants of 10 and 4 ms for cellulose and noncellulosic material, respectively, points to gathering of at least several cellulose crystallites in each microfibril, as in the model proposed by Frey-Wyssling (1954). MacKay et al. (1988) used similar reasoning to set a lower limit of 10 nm on the mean diameter of cellulose microfibrils in bean seedling hypocotyls.

Crystallinity. Frey-Wyssling (1954) suggested that the "astonishingly poor" crystallinity of cellulose in primary cell walls of corn could be the result of hemicelluloses and pectins hindering orderly crystallization. A broad cortex of paracrystalline chains was envisaged as surrounding each crystallite. These conclusions were based on X-ray diffraction patterns that showed most of the diffracted radiation spread in diffuse bands assigned to amorphous or paracrystalline material.

Jarvis (1990) seems to refer to a model similar to that of Frey-Wyssling in stating that "the large proportion of amorphous cellulose probably dominates the interaction of the microfibrils with non-cellulosic polymers bound to their surface". Jarvis used NMR spectroscopy but did not resolve fine structure in the band near 84 ppm and therefore assigned the band to "amorphous, or crystallite surface" material.

Proton spin relaxation time constants for amorphous cellulose would be expected to be similar to those for other amorphous carbohydrate polymers, e.g., hemicelluloses and pectin. Failure to detect signals from amorphous cellulose in Figure 2b therefore provides evidence for a

lack of amorphous cellulose in the sample of apple cell walls. This observation does not eliminate the possibility of partly ordered material clinging to crystal surfaces, since proton spin diffusion between the surface and the crystal interior could lengthen the proton rotating-frame spin relaxation time constant sufficiently for signals to appear in Figure 2a rather than Figure 2b. However, the PSRE subspectrum of well-ordered cellulose (Figure 2a) shows no obvious evidence for a broad background under the band near 85 ppm. The question of whether apple cell walls contain any partly ordered cellulose could perhaps be answered by combining PSRE and resolution-enhancement techniques, but data-averaging periods would be prohibitively long unless spectrometer sensitivity can be improved far beyond that available in the present work.

Our failure to find any evidence of amorphous cellulose is consistent with the proton NMR results of MacKay et al. (1988). Those authors measured the interpair second moments for protons in bean seedling hypocotyls and found a component with a second moment similar to the rigid-lattice value for cellulose. This observation led MacKay et al. (1988) to the conclusion that primary wall cellulose is at least 70% crystalline.

It is important to note a difference in the effects of order and disorder on X-ray diffraction patterns and NMR spectra. The X-ray method is sensitive to long-range order, e.g., repetition of cellulose chains at regular intervals through a crystallite. The NMR method is sensitive to short-range order, e.g., whether a cellulose chain is surrounded by similar chains or exposed on a surface. The NMR method therefore seems to provide a more reliable test of crystallinity in cases where typical crystallites are just a few molecules thick.

It is, of course, possible that plant material from different sources might contain different proportions of amorphous cellulose, and the apple cell wall cellulose studied in the present work might not be typical of cellulose in other primary cell walls.

Crystalline Forms. Signals at 88.2, 89.2, and 90.1 ppm in Figure 3 can be interpreted as a pair of overlapping doublets assigned to the monoclinic form (88.2 and 89.2 ppm, labeled "m") and triclinic form (89.2 and 90.1 ppm, labeled "t") (Atalla and VanderHart, 1984; VanderHart and Atalla, 1984). This interpretation depends on both crystalline forms having two chains per unit cell. It is generally agreed that there are two chains per monoclinic unit cell (Sugiyama et al., 1991). Triclinic unit cells with two chains have been suggested by Sarko and Muggli (1974) and Sugiyama et al. (1990), but more recent electron diffraction evidence points to the possibility of a single chain per unit cell (Sugiyama et al., 1991). Those results need to be substantiated by a refinement based on diffraction intensity data. Regardless of the detailed interpretation, it is clear that similarities in signal heights at 88.2 and 90.1 ppm point to similar proportions of the monoclinic and triclinic forms in the case of the apple cell walls (Figure 3a). The evidence is supported by relative strengths of signals assigned to C-1 in Figure 1b. A pair of signals at 104.3 and 106.3 ppm, assigned to the monoclinic crystalline form, are mere shoulders on a stronger signal at 105.5 ppm, assigned to the triclinic form plus chains on crystal surfaces (VanderHart and Atalla, 1984).

Celluloses of natural origin are generally placed in one of two families, the bacteria–*Valonia* family or the cotton–ramie family, characterized by relatively high or low proportions, respectively, of the triclinic crystalline form (Debzi et al., 1991). The cellulose of apple cell walls does not seem to fit neatly into either family.

Mechanical Properties. The nature and degree of molecular ordering of cellulose in primary cell walls could have interesting implications in accounting for mechanical properties. Poorly ordered cellulose is generally associated with properties of flexibility and toughness (Mark, 1954), properties that would not be desirable in an apple. Highly ordered cellulose is generally associated with properties of hardness and dimensional stability (Mark, 1954). The relatively narrow cross sections of crystallites in apple cell walls would make them relatively susceptible to mechanical damage when compared with, e.g., cellulose in wood. A combination of properties of hardness and weakness in the individual crystallites would contribute to the brittle, "crisp" texture of an apple.

The cellulose microfibrils are embedded in a noncellulosic matrix that includes pectin and protein networks. Any variations in the nature of this matrix must contribute to variations in the mechanical properties of cell walls. Molecular ordering of cellulose should therefore be considered as a contributing factor alongside other intermolecular interactions, e.g., within the pectin (Irwin et al., 1992). Our work complements other studies of apple cell walls by providing information about the nature of the cellulose, a topic that has not been as thoroughly researched in other studies of apple cell walls.

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