PERGAMON

PHYTOCHEMISTRY

Phytochemistry 51 (1999) 17–22

Polymer mobility in cell walls of cucumber hypocotyls

K.M. Fenwick[a], D.C. Apperley[b], D.J. Cosgrove[c], M.C. Jarvis[a,*]

[a]*Chemistry Department, Glasgow University, Glasgow G12 8QQ, UK*
[b]*EPSRC Solid-state NMR Unit, Mountjoy Research Centre, Durham University, Durham DH1 3LE, UK*
[c]*208 Mueller Laboratory, Department of Biology, Pennsylvania State University, University Park, PA 16802 USA*

Received in revised form 26 September 1998

Abstract

Cell walls were prepared from the growing region of cucumber (*Cucumis sativus*) hypocotyls and examined by solid-state ^{13}C NMR spectroscopy, in both enzymically active and inactivated states. The rigidity of individual polymer segments within the hydrated cell walls was assessed from the proton magnetic relaxation parameter, T_2, and from the kinetics of cross-polarisation from ^{1}H to ^{13}C. The microfibrils, including most of the xyloglucan in the cell wall, as well as cellulose, behaved as very rigid solids. A minor xyloglucan fraction, which may correspond to cross-links between microfibrils, shared a lower level of rigidity with some of the pectic galacturonan. Other pectins, including most of the galactan side-chain residues of rhamnogalacturonan I, were much more mobile and behaved in a manner intermediate between the solid and liquid states. The only difference observed between the enzymically active and inactive cell walls, was the loss of a highly mobile, methyl-esterified galacturonan fraction, as the result of pectinesterase activity.

Keywords: *Cucumis sativus*; Cucurbitaceae; Growth; Cell walls; Pectins; Microfibrils; Mobility; Solid-state NMR

1. Introduction

The expansion of plant cells, although driven by turgor pressure, is permitted and controlled by the enzymically-mediated loosening of the cell walls (Cosgrove, 1993). Much of the lateral strength and rigidity of dicot cell-walls is provided by a layered network of cellulose microfibrils, cross-linked by xyloglucans (Fry, 1986). Cell expansion is directed along the axis of the plant if the microfibrils are predominantly wrapped round the cells at right angles to that axis (Preston, 1974).

The loosening response of enzymically active cell walls can be detected in straightforward mechanical experiments on elongating plant stems. It has different characteristics from the conventional, viscoelastic behaviour of cell walls that lack active enzymes. Enzymic wall-loosening (creep) proceeds linearly with time for as long as a constant stress is applied and the requisite enzymes are active (Cosgrove & Durachko, 1994), whereas inactive, viscoelastic cell walls under constant stress approach an equilibrium extension exponentially. A new class of cell-wall proteins, the expansins, have been implicated in the loosening of the cell wall during growth (Cosgrove & Durachko, 1994; McQueen-Mason & Cosgrove, 1995; Cosgrove, 1996). They cleave hydrogen bonds between fibres of cotton cellulose in vitro (McQueen-Mason, 1995) but their natural substrate is at present unknown.

It is possible to estimate the rigidity of individual polymers within a composite structure, like a cell wall, by solid-state NMR relaxation methods (Stejskal & Memory, 1994; Foster, Ablett, McCann, & Gidley, 1996; Jarvis, Fenwick, & Apperley, 1996). Nuclear magnetisation can be dissipated, under a variety of different conditions, at rates that depend on the amount of thermal motion and, hence, on the rigidity of the polymer concerned. In this way, the contribution that each polymer makes to the rigidity or strength of the entire structure, can in principle, be assessed. This has been done for cell walls from a variety of plant tissues (e.g. Foster et al., 1996; Jarvis et al., 1996; Newman, Davies, & Harris, 1996), but not for any of the cell-wall systems used classically in the study of growth.

Herein, we describe NMR relaxation experiments on the cell walls of cucumber hypocotyls, the plant tissue first used for the isolation and testing of expansins. Under the conditions used, we could not identify any changes in molecular rigidity that could be attributed to expansin activity but, nevertheless the results serve as a baseline for understanding what determines the internal rigidity of dicot cell walls capable of loosening in order to allow growth.

* Corresponding author.

0031-9422/99/$ - see front matter
PII: S0031-9422(98)00723-7

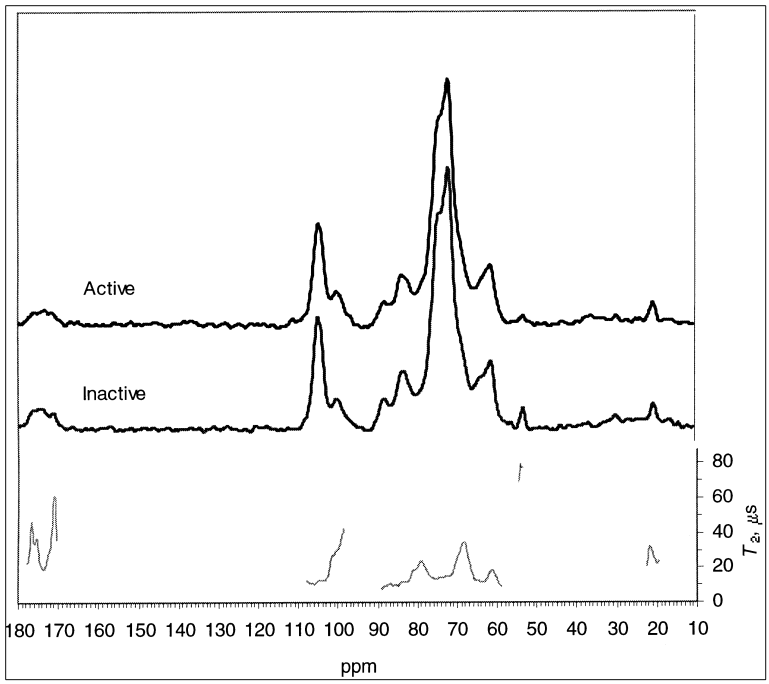

Fig. 1. CP-MAS ^{13}C NMR spectra of enzymically active and inactive cell walls from cucumber hypocotyls; see Table 1 for peak assignments. Bottom curve: proton spin–spin relaxation time T_2 plotted across the spectrum (gaps correspond to spectral regions where there was not enough signal intensity to permit its measurement). Long proton T_2 values indicate high mobility.

2. Results and discussion

The solid-state ^{13}C NMR spectrum of the cucumber hypocotyl cell walls is shown in Fig. 1 and Table 1 and is typical of those spectra from the cell-walls of dicots.

Table 1
Assignment of peaks in the ^{13}C NMR spectrum of cucumber cell walls

Chemical shift (ppm)	Assignment
177	C-6 of galacturonan with monovalent cation
175	C-6 of galacturonan in calcium form
173	carboxyl of acetyl
171	C-6 of galacturonan in H- or Me form
105	C-1 of cellulose, xyloglucan main chain and β(1-4) galactan
101	C-1, predominantly of galacturonan
89	C-4 of cellulose I
84	C-4 of crystallite-surface cellulose
82.5	C-4 of glucan chain in xyloglucan
80	C-4, predominantly of galacturonan in random-coil and 3_1 helical forms
75	general carbohydrate C-2, C-3, C-5
72	general carbohydrate C-2, C-3, C-5
69	C-2/C-3 of galacturonan
65	C-6 of cellulose I
62	C-6 of crystallite-surface cellulose, galactose in xyloglucan and β(1-4) galactan
53	pectic methoxyl
21	CH_3 of acetyl

There was no significant difference between the spectra from enzymically active and inactive cell walls, except for a reduction in the size of the pectic methyl ester peak at 54 ppm in the spectrum of the active cell walls.

It has been suggested that cell walls from growing plant tissues are characterised by a high proportion of branched and methyl-esterified pectic polysaccharides (Rees & Wight, 1969; Goldberg, Morvan, Jauneau, & Jarvis, 1996). This feature is not evident in the spectra shown. In particular, the peak corresponding to pectic methyl esters (54 ppm) was smaller in the spectrum from the active cell walls than that from inactive cell walls. In principle, this could result from an NMR problem; methyl-esterified galacturonan and pectic galactan chains are under-represented in conventional CP-MAS ^{13}C spectra of hydrated cell walls, because they are normally very flexible and exhibit so much thermal motion that they are cross-polarised only very slowly (Foster et al., 1996; Ha, Evans, Jarvis, Apperley, & Kenwright, 1996). Two procedures have been independently developed for recovering spectra from these highly mobile polymer segments. Either (a) CP is omitted and the more rigid components are edited out of the direct-polarisation ^{13}C spectrum by allowing insufficient time for ^{13}C T_1 decay (Foster et al., 1996) or (b) the slow CP of the mobile material is exploited to extract a difference spectrum from a combination of a delayed-contact and a long-contact experiment (Ha et al., 1996). Spectra obtained in these two quite different ways are in good agreement (Foster et al., 1996; Ha et al., 1996).

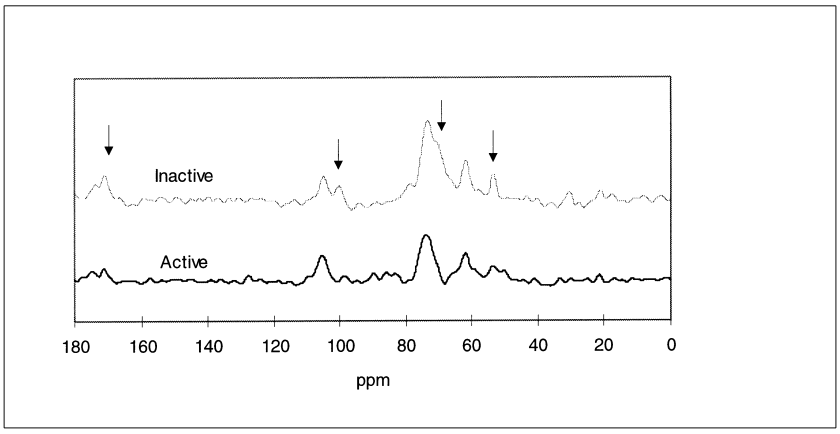

Fig. 2. Reconstructed ^{13}C NMR spectra of highly mobile regions of enzymically active and inactive cell walls from cucumber hypocotyls, cross-polarising between 1 and 7 ms; see Table 1 for peak assignments. Arrows mark the position of resonances assigned to methyl-esterified galacturonans. Total spectral intensity relative to Fig. 1 was 10% for the active cell walls and 13% for the inactive cell walls.

In the present work, the contact time was lengthened to bring as much as possible of the mobile material into the conventional CP-MAS spectra shown in Fig. 1. In addition, a simplified spectral editing procedure based on approach (b) above (Ha et al., 1996) was used to construct the spectra in Fig. 2, derived from polymer chain segments that were too mobile to appear in Fig. 1. The upper spectrum in Fig. 2 shows that mobile segments of galactan (C-1, 105 ppm; C-6, 62 ppm) and methyl-esterified galacturonan (C-1, 101 ppm; C-2/C-3, 69 ppm; C-6, 171 ppm; methyl, 54 ppm) were present in the enzymically inactive cell walls, in addition to the more rigid material observed in Fig. 1. In the active cell walls, the quantity of this mobile fraction was less and it included little or no galacturonan. The procedure used to derive the spectra in Fig. 2 is not expected to record all the material absent from Fig. 1 and the difference in intensity between Fig. 2(A) (active) and Fig. 2(B) (inactive) must be regarded with caution; however, there was no evidence for an *increase* in the abundance of any mobile fraction associated with enzymic activity.

In hydrated cell walls, the proton spin–spin relaxation time, T_2, increases with molecular motion on the kHz timescale (Ha et al., 1996; Newman et al., 1996). It can be measured through the ^{13}C spectrum using a pulse sequence developed by Tekely and Vignon (1987). Fig. 1 shows a spectral plot of the proton T_2 for hydrated cucumber cell walls. The spectral regions characteristic of cellulose and xyloglucan had proton T_2 values of the order of 10 μs, typical of highly rigid solids. The regions characteristic of pectin galacturonans and galactans had longer and very variable proton T_2 values, intermediate between those of solids and liquids. Spin–spin decay curves for cellulose were close to Gaussian in form, as expected theoretically for rigid solids, whereas those for pectic resonances were closer to exponential (Fig. 3). The spectral editing method of Newman, Ha, and Melton (1994) was used to derive subspectra corresponding to low and intermediate mobility from those wall polymers that were rigid enough to cross-polarise within 3 ms (Fig. 4). The 'rigid' subspectrum corresponds to cellulose and most of the xyloglucan, whereas the more mobile subspectrum includes a minor xyloglucan fraction and is otherwise exclusively pectic. This emphasises the sharp distinction in mobility between the rigid cellulose–xyloglucan microfibrils and the predominantly pectic matrix, as has been observed with other pectin-rich primary cell walls (Ha et al., 1996; Jarvis et al., 1996).

In mechanical extension experiments, the ability of the cell walls to respond to endogenous expansin activity is slowly lost over a period of some hours after the cell walls are rehydrated (McQueen-Mason, 1995). As a result, it was necessary to use relatively simple NMR experiments with a maximum duration of 5 h and to accept a lower signal-noise ratio than would otherwise be desirable.

Within these limitations, the experiments reported here showed no effect of enzymic activity in the cell walls, with the exception of the loss of pectic methyl esters and the consequent reduction in the mobility of the galacturonan segments carrying them. These effects may be attributed to pectinesterase activity and are not likely to be associated with the mechanism of growth, although they may be connected with the progressive loss of the capacity for growth over time. The relevance of the data presented here is to the way in which cell walls capable of growth are assembled from polymers of varying rigidity and not to the mechanism of cell-wall loosening that permits growth.

It is common to consider the growing cell wall as a composite material like fibreglass. On this basis, the cellulose–xyloglucan microfibrils (not merely cellulose itself) form the fibre phase and the matrix is largely pectic. Analogy with a composite material explains how the orientation of growth is directed by patterns of microfibril deposition (Preston, 1974) but it fails to explain how growth occurs. Extension of a conventional composite

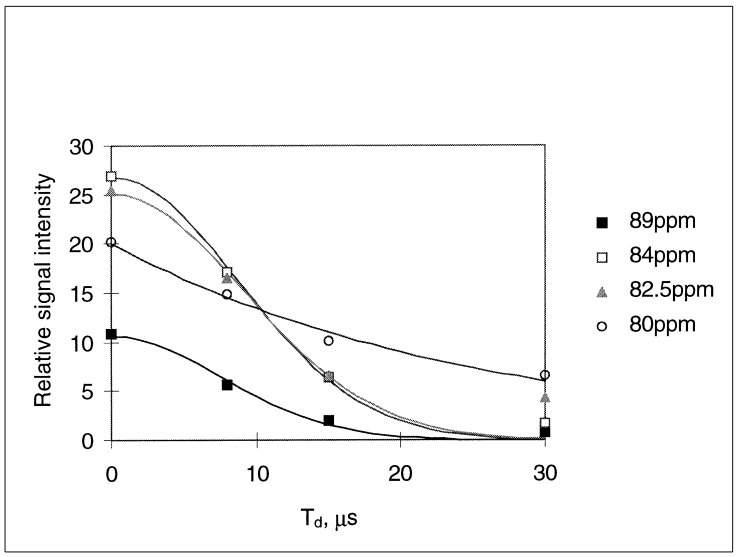

Fig. 3. Proton T_2 decay curves associated with C-4 peaks in the ^{13}C spectrum corresponding to different polysaccharides. Fitted curves are Gaussian for the 89 and 84 ppm peaks derived from cellulose and the 82.5 ppm peak from xyloglucan, exponential for the 80 ppm peak from galacturonan.

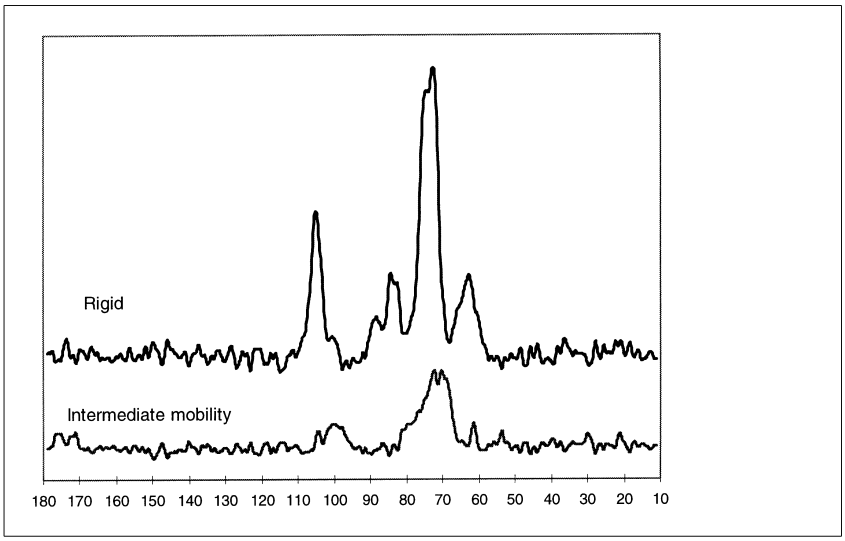

Fig. 4. CP-MAS ^{13}C NMR subspectra of cucumber hypocotyl cell walls, corresponding to short ('rigid') and long ('intermediate mobility') values of the proton T_2.

material depends on the extensibility of the fibres, their orientation relative to the stress, the bulk rigidity of the matrix and the adhesion between the matrix and the fibre surface (Harris, 1980). In the cell wall, the fibres are effectively inextensible and their ratio of length to diameter (>100) is so great that fibre–matrix adhesion is of no consequence. Extension, according to composite theory, would then require a significant decrease in the bulk rigidity of the matrix (Harris, 1980). No such effect was observed here.

The inadequacy of the 'fibreglass model' is also apparent in the very wide variation in the rigidity of polymer chains within the matrix. This was not merely a difference between the mobility of individual molecules within a structure homogeneous above the molecular scale; the matrix must have contained stiff and loose spatial domains, since pectic components differing measurably in proton relaxation properties must be separated by at least 2–3 nm to avoid averaging by proton spin diffusion (Newman et al., 1994; Ha, Evans, Apperley, & Jarvis, 1996). It seems more appropriate to regard the growing cell wall as a nanostructure, in which stresses between the microfibrils are carried, not by the matrix as a whole, but by a small number of polymer chains that are specific in structure and orientation. Examples of these might include a minor, stressed fraction of the hemicellulosic

cross-links (McCann & Roberts, 1994; Cosgrove, 1996) or the trace pectic component, rhamnogalacturonan II (Findeklee & Goldbach, 1996). We conclude that the load-bearing polymer chains in question either (1) are not abundant enough to be detected in the relaxation experiments described here or (2) are loosened in some way that is slower than the kHz thermal motions detected by these NMR experiments and is tightly controlled in direction.

We do not at present know enough about polymer architecture around and between the microfibrils nor about the way in which the microfibril network distorts when the cell expands, to understand the mechanism of cellular growth. Identification of the natural substrate for the expansins, which appear to cleave non-covalent bonds between cellulose-like molecules (McQueen-Mason & Cosgrove, 1995), would certainly bring progress in this direction.

3. Experimental

3.1. Cell wall preparation

Cell walls were prepared from the apical 60 mm of cucumber hypocotyls (*Cucumis sativus* L. cv. Burpee Pickler) that had been grown in darkness for 4 days, as described previously (Cosgrove & Durachko, 1994). For inactivation of expansins, cell walls were heat-treated in boiling water for 1 min. Enzymically active controls were not heat-treated. Cell walls were stored dry and rehydrated to 50% moisture content immediately before packing into the magic-angle spinning rotor. This relatively low moisture content was necessary in order to maximise the signal-noise ratio and reproducibility of the NMR experiments. Never-dried cell walls were not used because their moisture content could not be adjusted uniformly. It was established in control experiments (data not shown) that the low-pH creep response, characteristic of the activity of endogenous expansins, remained evident at this moisture content in dried and rehydrated hypocotyl segments.

3.2. NMR experiments

All NMR experiments were carried out at 75.43 MHz for ^{13}C using cross-polarisation and magic-angle spinning at 3 kHz (CP-MAS). The proton radiofrequency field was set at maximum power (60 kHz) and the ^{13}C field was adjusted to optimise the Hartmann–Hahn match for each sample individually. The initial proton 90° pulse was then re-optimised. These precautions were necessary to allow for variation in radiofrequency absorption by water protons. From the intensity of the ^{13}C field necessary to optimise the Hartmann–Hahn condition, it appeared that the proton field-strength reaching the cell-wall polysaccharides was less than half of that applied. The same proton radiofrequency field strength was maintained during decoupling.

Spectra from polymers of high mobility were obtained from an experiment in which the duration τ of the Hartmann–Hahn contact was varied from 100 μs to 12 ms. The spectra shown Fig. 2 were derived from linear combinations of the spectra averaged for the τ windows $\tau = 100$–1000 μs and $\tau = 5$–9 ms. They are therefore derived from polymer segments cross-polarising between these time intervals.

The proton spin–spin relaxation time T_2 was measured through the ^{13}C spectrum by inserting a delay T_d of 0–30 μs between the proton preparation pulse and the Hartmann–Hahn contact (Tekely & Vignon, 1987). A relatively long contact time of 3 ms was used in this experiment, since preliminary variable-contact experiments showed that the hydrated cell walls contained highly mobile pectic polysaccharides that required some ms for cross-polarisation; these experiments established 3 ms as the most suitable compromise between adequate cross-polarisation of this mobile material and minimal $T_{1\rho}$ decay of less mobile pectic polymers. Subspectra corresponding to rigid and intermediate-mobility components were obtained by taking linear combinations of the spectra at $T_d = 0$ and $T_d = 15$ μs.

References

Cosgrove, D. J. (1996). *BioEssays*, *18*, 533.

Cosgrove, D. J. (1993). *New Phytologist*, *124*, 1.

Cosgrove, D. J., & Durachko, D. M. (1994). *Journal of Experimental Botany*, *45*, 1711.

Findeklee, P., & Goldbach, H. E. (1996). *Botanica Acta*, *109*, 463.

Foster, T. J., Ablett, S., McCann, M. C., & Gidley, M. J. (1996). *Biopolymers*, *39*, 51.

Fry, S. C. (1986). *Annual Review of Plant Physiology*, *37*, 165.

Goldberg, R., Morvan, C., Jauneau, A., & Jarvis, M. C. (1996). In J. Visser & A. G. J. Voragen (Eds.), *Pectins and pectinases* (p. 166). Amsterdam: Elsevier.

Ha, M. A., Evans, B. W., Apperley, D. C., & Jarvis, M. C. (1996). In J. Visser & A. G. J. Voragen (Eds.), *Pectins and pectinases* (p. 561). Amsterdam: Elsevier.

Ha, M. A., Evans, B. W., Apperley, D. C. and Jarvis, M. C. (1996). In J. Visser & A. G. J. Voragen (Eds.), *Pectins and pectinases* (p. 561). Amsterdam: Elsevier.

Harris, B. (1980). In J. F. V. Vincent & J. D. Currey (Eds.), *The mechanical properties of biological materials* (p. 37). Cambridge: Cambridge University Press.

Jarvis, M. C., Fenwick, K. M., & Apperley, D. C. (1996). *Carbohydrate Research*, *288*, 1.

McCann, M. C., & Roberts, K. (1994). *Journal of Experimental Botany*, *45*, 1683.

McQueen-Mason, S. J. (1995). *Journal of Experimental Botany*, *46*, 1639.

McQueen-Mason, S. J., & Cosgrove, D. J. (1995). *Plant Physiology*, *107*, 87.

Newman, R. H., Davies, L. M., & Harris, P. J. (1996). *Plant Physiology*, *111*, 475.

Newman, R. G., Ha, M. A., & Melton, L. D. (1994). *Journal of Agricultural and Food Chemistry*, *42*, 1402.

Preston, R. D. (1974). *The physical biology of plant cell walls*. London: Chapman and Hall.

Rees, D. A., & Wight, N. J. (1969). *Biochemical Journal*, *115*, 431.

Stejskal, E. O. & Memory, J. D. (1994). *High resolution NMR in the solid state*. *Fundamentals of CP/MAS*. New York: Oxford University Press.

Tekely, P., & Vignon, M. R. (1987). *Journal of Polymer Science Part C: Polymer Letters*, *25*, 257.