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Anomalous proton NMR relaxation behavior of cell wall materials from Chinese water chestnuts

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ABSTRACT: Proton T_1 NMR relaxation of the cell wall materials from Chinese water chestnuts (CWC) is an order of magnitude more efficient than those from potatoes, even though both the major relaxation pathways and the relative proton population of the relaxation groups are similar. This anomalous behavior of CWC can be explained by the detection of a strong ESR signal, which is an order of magnitude larger than that in samples from potatoes. The estimated free radical concentration is about 60 μM. Spectral properties of the radicals ($g = 2.00599$, linewidth 10.75 G) are consistent with that of semiquinone radicals derived from wall-bound polyphenols in cell wall materials. Hydration led to the drastic reduction of the ESR signal; it virtually disappeared when as little as 13% water was added to CWC in the vapor phase. Redrying over phosphorus pentoxide under vacuum followed by exposure to air resulted in some recovery of the free radical signals. Paradoxically, ^{1}H T_1 relaxation of a wet CWC (21% D_2O) showed sustained efficiency at $T > 300$ K. $T_{1\rho}$ relaxation was only moderately affected by the presence of free radicals and transverse relaxation showed no effects. All the relaxation behavior could be accounted for by a spin diffusion model involving low concentration of unpaired electron spins. Copyright © 2000 John Wiley & Sons, Ltd.

KEYWORDS: NMR; ESR; proton relaxation; plant cell walls; potato; Chinese water chestnut; free radicals

INTRODUCTION

Plant cell walls, consisting of cellulose, hemicellulose, pectin, a small fraction of proteins and polyphenols,[1] play a vitally important part not only in many aspects of living activities of plant cells but also in digestive processes of foods as so-called dietary fibers. In addition, they are of significance in physical properties of many vegetables and fruits. For example, the texture of vegetable foods has been thought to be related to the cell walls of the plants.[2–4] The differences in texture perception of cooked potatoes and Chinese water chestnuts[2,5–7] has been thought to be related to the higher content of phenolic components of the cell wall of the latter.[5] Phenolic components can act as antioxidants[8,9] as well as free radical-generating centers.[10]

In our systematic NMR investigations on the molecular dynamic properties of the plant cell wall materials[11–14] and model systems[11,15] (H. R. Tang, P. S. Belton, S. C. Davies and D. L. Hughes, unpublished results), we found that the motions of methyl and methoxyl groups have a frequency close to the Larmor frequency (10^8 Hz) below 200 K,[11,15] whereas the motions of exchangeable protons, including hydroxyl groups and water, and hydroxymethylene groups reach that frequency at temperature well above 350 K.[11,13,15] We also observed that the proton spin–lattice relaxation (T_1) was much more efficient for cell wall materials from Chinese water chestnuts than that from potatoes,[11] although the concentration of both the hydroxyl groups and hydroxymethylene groups in the Chinese water chestnut cell walls was similar to that in potatoes.[12] In this paper we report on an observation of a large ESR signal from the cell wall materials of Chinese water chestnut and explain the proton relaxation behavior in terms of the interaction of the nuclear spins with these electron spins.

EXPERIMENTAL

Cell wall materials of potatoes (PB) and Chinese water chestnuts (CWC) were extracted according to a method described previously[2,13] and prepared as described before.[13] TEMPO (2,2,6,6-tetramethylpiperidine-*N*-oxyl) was purchased from Sigma. Hydration of cell wall materials was carried out in the vapor phase over water in a sealed container at ambient temperature. The level of hydration is expressed as a percentage on the weight to weight basis (e.g. 21% means 21 g of water per 100 g of solid). Drying was conducted in a desiccator over P_2O_5 under vacuum.

^{1}H relaxation times were measured on a Bruker MSL-100 spectrometer with a 5 mm proton-dedicated probe. Temperature was controlled with a Bruker B VT-1000 variable temperature unit equipped with a titanium thermocouple. Liquid nitrogen was boiled off for temperatures lower than the ambient temperature while compressed air was used for temperatures above ambient. All measurements were conducted from the lowest temperature

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with an increment of 5–10 K. Typically, temperature was decreased from 298 to 200 K within 2 min. A duration of 15 min was used to allow the sample to reach constant temperature before measurements were made.

Spin–lattice relaxation times, T_1 and $T_{1\rho}$, were measured using standard inversion–recovery (180–τ–90–aq) and spin-locking sequence (90–$\tau_{\text{Spin-Lock}}$–aq). For the former, 48 delays, τ, were chosen with a logarithmic function as described previously[16] so that multi-exponential processes can be detected with ease. For $T_{1\rho}$, 48–128 delays, $\tau_{\text{Spin-Lock}}$, were chosen with a linear increment. Single point sampling was performed 5–10 μs after the detection pulse. The recycle delay in both experiments was chosen to be longer than five times T_1. The residual second moment was measured by fitting the decay part of a solid echo using a modified Gaussian function[13,17] with a dwell time of either 0.5 or 1 μs.

ESR spectra were recorded on a Varian-4 ESR X-band spectrometer with samples contained in a 5 mm NMR tube. For some experiments, a 5 mm sample tube fitted with a greaseless stopcock was used. The microwave power used was 1 mW unless described otherwise. The other parameters used were 93 kHz modulation with 5 G modulation amplitude and a sweep width of 200 G. The concentration of free radicals was estimated using a sample of powdered coal with a known concentration of free radicals (2.14×10^{-4} M) as standard.

RESULTS AND DISCUSSION

ESR spectroscopy

The cell wall materials from both potatoes and Chinese water chestnuts were studied with ESR spectroscopy, and for the convenience of discussion, the samples are referred to as PB and CWC respectively, in the following text. Figure 1 shows some signals observed in the ESR spectra for both PB and CWC in the form of vacuum-dried powder from freeze-dried materials. Under the same conditions, a relatively large ESR signal was detected for CWC [Fig. 1(B)] whereas a small but visible signal was detectable for PB [Fig. 1(A)]; the signal intensity of CWC is about an order of magnitude greater than that of PB. The single broad signal (linewidth 10.75 G) and a high g value (2.00599) suggest that the free radical is probably oxygen centered. The signal for PB is too weak to allow the determination of these parameters. ESR signals for both PB and CWC samples stored under vacuum for 2 months did not show any reduction of intensity, indicating the stability of the free radicals.

The first observation of free radicals in dry biological tissues was made in 1954[18] and there have been many similar observations thereafter in both animal and plant tissues, especially when freeze-dried.[10] Their presence has been attributed to reactions between oxygen and lyophilized tissues.[10] ESR signals have also been observed in dry model biological systems[10] and a number of irradiated foods.[19] However, no report appears to have considered free radicals in the prepared plant cell wall materials. This observation is significant since prepared cell wall materials represent 'purified' systems, in which both cell contents and water soluble wall components have been removed.[1] ESR signals in CWC cannot be assigned to Mn(II) since the spectral properties are not consistent with those of Mn(II),[19] nor is it to be expected that Mn(II) would remain after the cell wall extraction process, which involved a chelation step.[2,13] The signal is more likely to originate from the presence of semiquinone radicals formed from polyphenols.[10] Since the cell wall purification process involves extensive washing to remove not only cell contents but also water-soluble cell wall components,[1] any quinone free radicals observed in the prepared cell wall materials would have to be generated from *wall-bound* polyphenols.

In our system, the difference in signal intensity for CWC and PB must be due to chemical structural differences between them since both samples were prepared and measured under identical conditions. One of the most obvious differences between them is the amount of polyphenols such as ferulic acid derivatives.[5] It has been reported that semiquinone radicals could be generated by mixing and freeze-drying polyphenols such as ethyl gallate (3,4,5-trihydroxybenzoate) with cellulose, but polyphenols alone failed to form radicals.[10] Therefore, the free radicals in CWC are probably semiquinone radicals produced by the reactions between polyphenols and the polysaccharide matrix of the cell walls.[10]

To examine the properties of the ESR spectra, we measured the saturation power of the CWC signal. Figure 2 shows the ESR signal intensity as a function of the

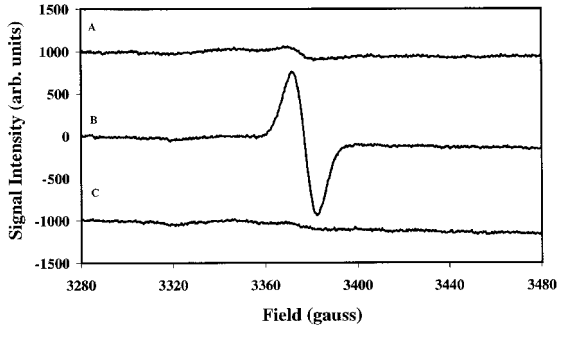

Figure 1. ESR spectra of plant cell wall materials. (A) Potatoes; (B) Chinese water chestnuts; (C) baseline.

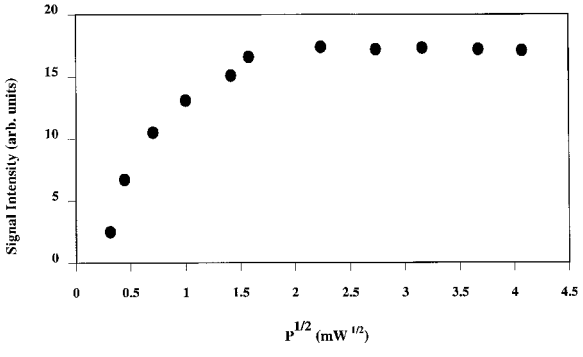

Figure 2. ESR signal intensity of cell walls of Chinese water chestnut as a function of microwave power.

square-root of microwave power.[20] It is apparent that the saturation power is approximately 2–2.5 mW. The appearance of a plateau above 2 mW suggests that the ESR lines are inhomogeneously broadened.[20] The microwave power (1 mW) used in recording these spectra is below the saturation power.

Figure 3 shows the hydration effects on the ESR signal intensity of CWC dried from H_2O. What is striking is that the ESR signals disappear when the hydration level is as low as 13% (13 g of water per 100 g of solid) [Fig. 3(B)]. Subsequent drying over P_2O_5 under vacuum followed by exposure to air, however, resulted in reappearance of the signals, although they were less strong [Fig. 3(C)] than the original ones [Fig. 3(A)]. D_2O-exchanged dry CWC samples also showed the same behavior. Some more CWC samples were tested with H_2O contents of 15, 17, 23 and 38%. In all cases, the wet samples showed a complete signal loss and drying over P_2O_5 under vacuum followed by exposure to air led to reappearance of the signals. We did not follow the hydration effects on PB signals owing to the weakness of its original signals. Nevertheless, a sample of PB boiled in D_2O for 30 min and then freeze-dried and vacuum-dried over P_2O_5 failed to show any increase of ESR signal intensity. This suggests that the ESR signals of CWC cannot be a consequence of heating.

Why did hydration have such detrimental effects on the ESR signals of CWC? The most likely reason for the signal loss is that free radicals have been destroyed by the hydration. To test these hypotheses further, we investigated the system containing PB and some added stable free radicals, TEMPO.

In order to understand the effect of hydration on the ESR signals of CWC, we added the stable free radical TEMPO to PB. Figure 4 shows the hydration effects on ESR signals of PB with added TEMPO. When the radicals were added in methanol solution, followed by removal of solvents, an intense 'powder' spectrum was observed [Fig. 4(A)]. Hydration over H_2O to about 27% did reduce the signal intensity to some extent but did not lead to disappearance [Fig. 4(B)]. Re-drying over P_2O_5 under vacuum did not recover the original signal intensity [Fig. 4(C)] as was the case with CWC. Nevertheless, there

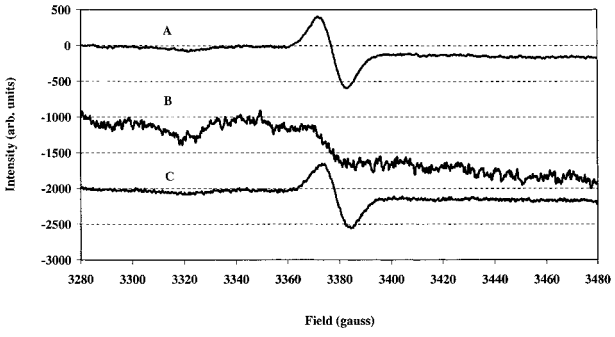

Figure 3. Hydration effects on ESR signals of cell wall materials of Chinese water chestnut. (A) Dry sample (receiver gain, 4000); (B) containing 13% water (receiver gain, 10 000); (C) after drying over P_2O_5 under vacuum (receiver gain, 4000).

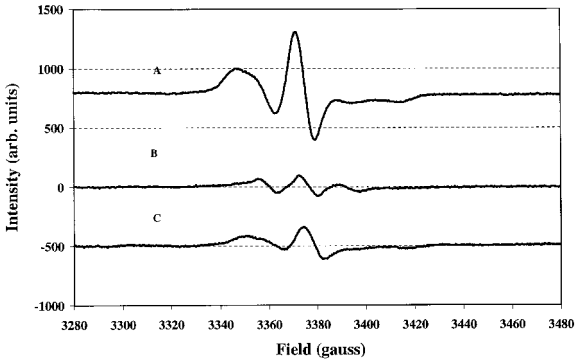

Figure 4. Hydration effects on ESR signals of potato cell walls containing TEMPO. (A) Dried sample; (B) containing 27% water; (C) after drying over P_2O_5 under vacuum.

was some small signal recovery. This implies that the signal loss in the wet CWC samples cannot be wholly explained by water interference with microwave power.

A CWC sample hydrated to 32% water was dried using vacuum pumping and showed only a small ESR signal recovery immediately after drying. When this sample was exposed to air for 3 days, the ESR signal recovered almost completely. This implies that hydration led to destruction of radicals and that oxygen must play an important role in the radical regeneration. However, it is not clear whether radicals are only partially or completely destroyed due to hydration.

Proton T_1 relaxation

T_1 relaxation of the cell wall materials of Chinese water chestnuts was found to be double-exponential over the temperature range 100–360 K[11,14] and the long component was designated T_{1L} and the short component T_{1S}. Similar multiple-component relaxation behavior was also observed for cell wall materials from PB.[11,13,14] This can be explained to be the typical heterogeneity of the plant cell wall assembly, in which spin diffusion is not efficient enough to achieve a common spin temperature on the time-scale of proton T_1. ^{13}C cross-polarization magic angle spinning (CP/MAS) studies showed[14] that the T_{1L} component was associated mostly with cellulose whereas the T_{1S} component was associated with non-cellulose polysaccharides. Since the T_{1L} component accounted for more than two-thirds of the total relaxation, we shall deal with mainly the T_{1L} in the following discussion.

It has been shown that the major relaxation processes for sugars in cell walls are reorientation of hydroxymethylene groups, by *trans–gauche* exchange,[11,13,21–23] and motions of exchangeable protons.[11,13,15,21–23] In the D_2O-exchanged sample, exchangeable protons are mostly replaced by deuterons, therefore the hydroxymethylene groups are the major remaining contributors. Figure 5 shows the rate of T_{1L} relaxation, R_{1L}, for CWC as a function of both temperature and hydration levels. It is apparent that R_{1L} of CWC increases as temperature increases, indicating a maximum at the high temperature, although this is not reached at 360 K. This confirms the relaxation

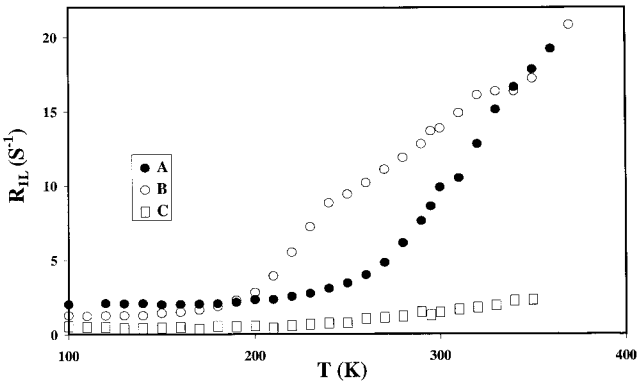

Figure 5. Proton spin–lattice relaxation rate in the laboratory frame for D_2O exchanged CWC and PB. (A) Dry sample; (B) containing 21% D_2O; (C) dry PB.

process by the reorientation of hydroxymethylene groups as discussed previously.[11,13,21–23] Hydration to about 21% D_2O led to an increase of R_{1L} at 200–330 K [Fig. 5(B)] but little change above 330 K. This hydration effect on T_1 can be explained in a way similar to that on PB.[11,13] However, it is also apparent that the values of R_{1L} for D_2O exchanged CWC at $T > 300$ K is up to an order of magnitude larger than that for PB [Fig. 5(C)] under similar conditions.[11] A monosaccharide analysis showed that the population of the protons from the hydroxymethylene groups relative to the total proton population in CWC was similar to that in PB.[12] If the same relaxation mechanism were solely responsible for the relaxation, this surprising observation could not be explained by the differences in the number of the relaxing groups. Therefore, some other extremely efficient relaxation mechanisms are expected to be responsible. The observation of ESR signals in this sample implies that the paramagnetic species offers an efficient relaxation pathway.

In the case where the paramagnetic species is dilute and there is efficient spin diffusion, the proton T_1 relaxation rate can be considered as the sum of two contributions from proton dipole–dipole interactions, R_{1d}, and from paramagnetic species, R_{1e}, and therefore

$$R_{1obs} = R_{1d} + R_{1e} \tag{1}$$

where

$$R_{1d} = \frac{3}{10}\frac{1}{N}\left(\frac{\mu_0}{4\pi}\right)^2 \gamma_p^4 \hbar^2 \sum_k r_{jk}^{-6} \times \left(\frac{\tau_c}{1+\omega_p^2\tau_c^2} + \frac{4\tau_c}{1+4\omega_p^2\tau_c^2}\right) \tag{2}$$

R1e can be written as[24]

$$R_{1e} = \frac{1}{10}\frac{1}{N}\left(\frac{\mu_0}{4\pi}\right)^2 \gamma_p^2\gamma_e^2\hbar^2 \sum_p r_{ep}^{-6}\left[\frac{\tau_c}{1+(\omega_e-\omega_p)^2\tau_c^2} + \frac{3\tau_c}{1+\omega_p^2\tau_c^2} + \frac{6\tau_c}{1+(\omega_e+\omega_p)^2\tau_c^2}\right] \tag{3}$$

where N is the total number of protons in the system, μ_0 the permeability of a vacuum, γ_p and γ_e the magnetogyric ratios of proton and electron, respectively, r_{jk}, r_{ep} the inter-spin distances between protons and between electron and protons and ω_p and ω_e the Larmor frequency for proton and electron. Since γ_e is about 658 times γ_p and ω_e is about 658 times ω_p, R_{1e} can be approximately rewritten as

$$R_{1e} = \frac{1}{10}\frac{1}{N}\left(\frac{\mu_0}{4\pi}\right)^2 \gamma_p^2\gamma_e^2\hbar^2 \sum_k r_{ep}^{-6} \times \left(\frac{3\tau_c}{1+\omega_p^2\tau_c^2} + \frac{7\tau_c}{1+\omega_e^2\tau_c^2}\right) \tag{4}$$

At $T > 350$ K, assuming that the motions of CH_2 groups are associated with the relaxation process, the maximum contribution of R_{1d} can be estimated[11] to be about $1–2\,s^{-1}$. Then the estimated R_{1e} would be about $18\,s^{-1}$. Although the free radical concentration in CWC was only about 60 μM, γ_e^2 is about 4×10^5 times γ_p^2, hence it is not surprising that R_{1e} is the dominant factor in the total relaxation. In a system dominated by the contribution from the free radicals, R_1 is expected to be closely dependent on the free radical concentration. Therefore, far more efficient relaxation in CWC than in PB can be explained by the observation that the ESR signal intensity in CWC is about an order of magnitude greater than in PB.

On hydration, if free radicals were destroyed completely, the proton relaxation efficiency resulting from free radicals would be eliminated. Our experimental results on a D_2O-hydrated CWC sample (Fig. 5) showed that this was not the case. This implies that some of the free radicals causing fast proton relaxation are still present in the sample.

Proton $T_{1\rho}$ and T_2 relaxation

$T_{1\rho}$ is sensitive to the motions comparable to the frequency of the spin-locking field ($10^4–10^5$ Hz).[25] Two components were observed for CWC. Results from ^{13}C CP/MAS and proton relaxation induced spectral-editing experiments showed that the long component, $T_{1\rho L}$, was associated mainly with cellulose and the short component, $T_{1\rho S}$, was associated with non-cellulose polysaccharides.[12,14] Figure 6 shows the relaxation rate of the long component, $R_{1\rho L}$, as a function of temperature, water content and spin-locking field. Peaks at about 260 K for the dry sample (40 kHz) [Fig. 6(A)] and at 200 K for the sample containing 21% D_2O (42 kHz) [Fig. 6(B)] are apparent. This shift of the maximum towards lower temperature is similar to that observed in potato samples.[11,13] This peak is associated with motional processes of hydroxymethylene groups and the effects of water plasticisation rather than freezing are thought to be responsible.[11,13] Moreover, for the wet CWC sample, $R_{1\rho L}$ showed a dependence on spin-locking field on both sides of the relaxation maxima [Fig. 6(B)–(D)], implying a distribution of correlation times for the motions.[11,13]

Similarly to R_1, when unpaired electron spins coupled to protons, the proton $R_{1\rho}$ can be considered as the

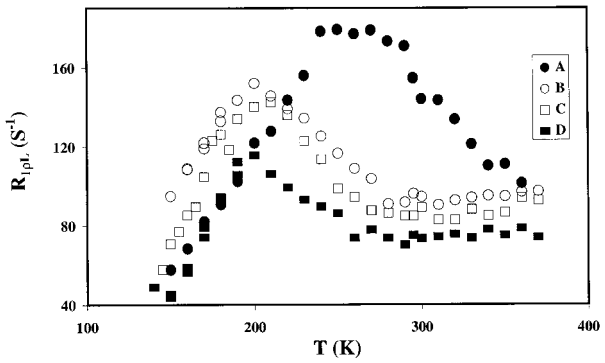

Figure 6. Proton spin–lattice relaxation rate in the rotating frame for CWC. (A) Dry sample, spin-locking field 40 kHz; (B) containing 21% D_2O, spin-locking field 42 kHz; (C) containing 21% D_2O, spin-locking field 67 kHz; (D) containing 21% D_2O, spin-locking field 100 kHz.

sum of both contributions from the proton dipole–dipole interactions,[24] $R_{1\rho d}$, and from paramagnetic species, $R_{1\rho e}$:

$$R_{1\rho obs} = R_{1\rho d} + R_{1\rho e} \tag{5}$$

where

$$R_{1\rho d} = \frac{1}{20}\frac{n_p}{N}\left(\frac{\mu_0}{4\pi}\right)^2 \gamma_p^4 \overline{h}^2 \sum_k r_{jk}^{-6}\left(\frac{4\tau_c}{1+4\omega_{eff}^2\tau_c^2} + \frac{5}{3}\frac{\tau_c}{1+\omega_p^2\tau_c^2} + \frac{2}{3}\frac{4\tau_c}{1+4\omega_p^2\tau_c^2}\right) \tag{6}$$

and

$$R_{1\rho e} = \frac{1}{20}\frac{n_e}{N}\left(\frac{\mu_0}{4\pi}\right)^2 \gamma_p^2\gamma_e^2 \overline{h}^2 \sum_p r_{ep}^{-6}\left[\frac{4\tau_c}{1+\omega_{eff}^2\tau_c^2} + \frac{\tau_c}{1+(\omega_e-\omega_p)^2\tau_c^2} + \frac{3\tau_c}{1+\omega_p^2\tau_c^2} + \frac{6\tau_c}{1+(\omega_e+\omega_p)^2\tau_c^2} + \frac{6\tau_c}{1+\omega_e^2\tau_c^2}\right] \tag{7}$$

where ω_{eff} is the effective relaxation field. Since ω_{eff} is generally of the order of tens of kHz whereas the Larmor frequency for proton and electron, ω_p and ω_e, are of the order of hundreds of MHz in our study, the above expressions can be simplified by approximation as follows:

$$R_{1\rho d} = \frac{1}{5}\frac{n_p}{N}\left(\frac{\mu_0}{4\pi}\right)^2 \gamma_p^4 \overline{h}^2 \sum_k r_{jk}^{-6}\left(\frac{\tau_c}{1+4\omega_{eff}^2\tau_c^2}\right) \tag{8}$$

$$R_{1\rho e} = \frac{1}{5}\frac{n_e}{N}\left(\frac{\mu_0}{4\pi}\right)^2 \gamma_p^2\gamma_e^2 \overline{h}^2 \sum_p r_{ep}^{-6}\left(\frac{\tau_c}{1+\omega_{eff}^2\tau_c^2}\right) \tag{9}$$

It is apparent that $R_{1\rho}$ is largely dependent on the effective relaxation field, which in turn depends on the actual spin-locking field and local static dipolar field.[11,13]

It might be expected that there would be a strong effect of the unpaired electron spins on the proton $R_{1\rho}$ relaxation. However, this is clearly not the case and $R_{1\rho}$ of CWC is not drastically different from that of PB.[11] For example, for the dry CWC, the $R_{1\rho}$ maximum (40 kHz) [Fig. 6(A)] at about 260 K is about 170–180 s^{-1}, which is only slightly larger than that of PB (160–170 s^{-1})[11] under similar conditions; for the CWC sample containing about 21% D_2O, the $R_{1\rho}$ maximum (67 kHz) [Fig. 6(C)] at about 200 K was about 150–160 s^{-1}, being slightly larger than that of PB (90–100 s^{-1}).[11,13] Consideration of the requirements of spin-locking indicates why this is so. In a system of dilute unpaired electron spins, only the nuclei close to the electrons are greatly affected by the dipolar interactions with them. Nuclei more remote are only affected via spin diffusion. When a spin-locking pulse is applied, spins remote from the unpaired electrons will be spin locked, but those close will have linewidth enhanced by the order of γ_e/γ_p. This will result in a failure to spin lock these protons. Protons adjacent to the unpaired electrons are thus not coupled in the rotating frame to those more remote, hence they cannot cause relaxation of the remote protons in the rotating frame by coupling to electrons. However, spin diffusion and relaxation can still take place in the laboratory frame. Hence enhancement of $R_{1\rho}$ by R_{1e} can still occur as transitions between Zeeman levels in the laboratory frame are a source of relaxation in the rotating frame.

T_2 relaxation processes of CWC were similar to that of potatoes.[11,13] The values of the second moment showed an overall 4 G^2 reduction [Fig. 7(A)] when the sample temperature was increased from 100 to 360 K. The occurrence of such a reduction of M_{2r} centered at about 250 K, which coincided with the $R_{1\rho}$ maximum (40 kHz), suggested that this was associated with the motional averaging of the static dipolar interaction of the hydroxymethylene groups. In the wet CWC sample, the M_{2r} reduction of 4 G^2 [Fig. 7(B)] was centered at about 180–190 K, which was also close to the $R_{1\rho}$ maximum. Effects due to unpaired electron spins are not observed since spin diffusion is slower than the transverse relaxation process.

In summary, the anomalous behavior of T_1 relaxation of CWC is due to the presence of semiquinone free radicals derived from the polyphenolic components. The effects of free radicals on the other relaxation processes of CWC are minimal. The effects of temperature and hydration on CWC relaxation are similar to that on PB. However, the observation of free radicals in cell walls is significant since the free radicals can affect NMR observations greatly, especially those studies depending on relaxation

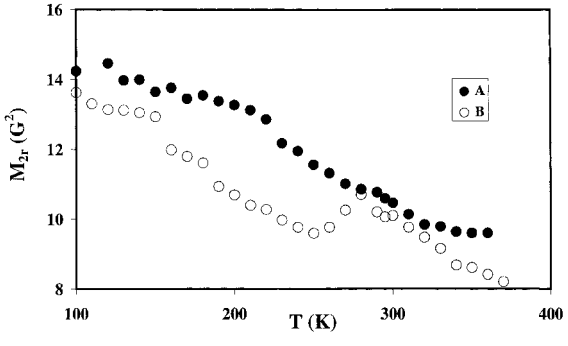

Figure 7. Proton residual second moment data for CWC. (A) Dry sample; (B) containing 21% D_2O.

properties. The presence of stable free radicals in foods could affect measurements on irradiated foods and could be undesirable in plants which have been developed to increase the phenolic content.

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