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Evidence for cross-linking in tomato cutin using HR-MAS NMR spectroscopy

Ashish P. Deshmukh[a,1], André J. Simpson[b], Patrick G. Hatcher[a,*]

[a]Department of Chemistry, The Ohio State University, 100, W. 18th Avenue, Columbus, OH 43210, USA
[b]Department of Physical and Environmental Sciences, The University of Toronto, Scarborough College,
1265 Military Trail, Toronto, Ontario, Canada M1C 1A4

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Abstract

Cutin is a polyester biopolymer component of plant leaf and fruit cuticles, most often associated with waxes and cuticular polysaccharides, and sometimes with another aliphatic biopolymer called cutan. Insolubility of these cuticular biopolymers has made it difficult to apply traditional analytical techniques for structure determination, because most techniques providing molecular level details require solubility. By using the relatively new technique of one and two-dimensional high-resolution magic angle spinning (HR-MAS) NMR spectroscopy, with added information from solid-state ^{13}C NMR spectroscopy, detailed through-bond connectivities and assignments are made for cutin from *Lycopersicon esculentum* (tomato) fruit. Based on the data obtained, tomato cutin is found to be predominantly an aliphatic polyester with some olefinic and aromatic moieties, consistent with previous studies that employed various degradative approaches. Aside from esters, there are free primary and secondary alcohol groups, as well as free fatty acids. A significant finding is the presence of α-branched fatty acids/esters. Mid-chain hydroxyls appear to be generally unesterified, but esters of mid-chain hydroxyls have been identified. The α-branched fatty acids/esters and esters of mid-chain hydroxyls could point towards cross-linking.

Keywords: Keywords; *Lycopersicon esculentum*; *Solanaceae*; Tomato; HR-MAS NMR spectroscopy; Esters; Branched carboxylic acids

1. Introduction

The two major polymeric lipid components found in plant cuticles are cutin and cutan. Whereas cutin is the polyester biopolymer that is solubilized upon saponification treatment, cutan is the non-saponifiable and non-extractable polymer found in certain cuticles. These polymers not only provide a protective barrier between the plant and its external environment, but also between different organs of the plant (Kolattukudy, 1980). While some cuticles have only cutin, and no cutan (e.g., *Lycopersicon esculentum* i.e. tomato fruit), others have only cutan (e.g., *Beta vulgaris*) and yet others may have both (e.g., *Agave americana* leaf) (Jeffree, 1996).

Cutin forms a major component of peels of fruits such as tomato and apple. Depolymerization of cutin using reactions such as alkaline hydrolysis, transesterification, and hydrogenolysis with $LiAlH_4$ in THF, gives monomers that are identified by gas chromatography-mass spectrometry (GC/MS) after derivatization (Kolattukudy, 1980). Such degradative studies suggest that cutin has a primary architecture comprised of C_{16} and C_{18} fatty acids, hydroxy fatty acids, and epoxy fatty acids (Fig. 1, Kolattukudy, 1980). Although there is mostly indirect evidence, extensive ester cross-linking of these monomeric species gives cutin its three-dimensional architecture (Kolattukudy, 1984). Concentrations of various monomers are found to be dependent on the species (Holloway, 1984), and the age of the cuticle (Riederer and Schonherr, 1988).

Various depolymerization protocols have indicated that the major monomers in tomato cutin are 10,16-dihydroxyhexadecanoic acid (more than 70%) and 16-hydroxyhexadecanoic acid (Baker and Holloway, 1970; Baker et al., 1982; Osman et al., 1995, 1999; Graca et al., 2002). Minor quantities of the corresponding 10-hydroxy and 10,18-dihydroxyoctadecanoic acids, and

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* Corresponding author. Tel.: +614-688-8799; fax: +614-688-4906.
E-mail address: hatcher@chemistry.ohio-state.edu (P.G. Hatcher).

[1] Present address: Department of Geosciences, Princeton University, 159 Guyot Hall, Princeton, NJ 08544, USA.

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the 9,10,ω-trihydroxy C_{16} and C_{18} acids are also observed. In addition, glycerol was recently found to be a component of tomato cutin (Graca et al., 2002). In studies of tomato cutin by our research group using thermochemolysis with tetramethylammonium hydroxide, the major products obtained were 10,16-dimethoxyhexadecanoic acid methyl ester (65%), and 16-methoxyhexadecanoic acid methyl ester, aside from *para*-coumaric acid methyl ester (del Rio et al., 1998; del Rio and Hatcher, 1998). Minor amounts of 8, 16- and 9, 16-dimethoxyhexadecanoic acid methyl esters, were also obtained. These results were in line with the findings of various depolymerization reactions carried out earlier (Baker and Holloway, 1970; Baker et al., 1982; Osman et al., 1995).

Degradative studies have a drawback in that they are invasive and as such, are usually able to allow only inferences about cross-linking and other kinds of connectivities between monomers. Mass spectrometry has been used to determine structures of oligomers obtained by enzymatic hydrolysis of lime cutin using porcine pancreatic lipase enzyme, which selectively cleaves esters of primary alcohols while possibly retaining some of the linkages (Ray and Stark, 1998; Osman et al., 1999). Based on the fragmentation patterns, it was shown that possible monomeric structures are the same as those obtained from complete hydrolysis (Ray and Stark, 1998). Ray et al. (1998) isolated four oligomers from lime cutin by selective hydrolysis of hindered esters by iodotrimethylsilane. The soluble oligomers were then characterized using homonuclear and heteronuclear solution state NMR spectroscopic techniques, and confirmation was obtained from mass spectrometric fragmentation patterns. Oligomers obtained from such selective hydrolysis treatments are not necessarily representative of the whole biopolymer. Furthermore, some of the monomers generated by complete depolymerization may not be amenable to gas chromatographic analysis with or without derivatization, and hence may escape detection. Insolubility of the cuticular biopolymers has made it difficult for solution-state NMR spectroscopic techniques to be applied for structure determination.

Solid-state nuclear magnetic resonance (NMR) spectroscopy has been used for structural characterization of intact lime cutin (Zlotnik-Mazori and Stark, 1988; Pacchiano et al., 1993; Fang et al., 2001). Cross-polarization magic angle spinning (CPMAS) NMR spectroscopy in combination with Bloch decay experiments confirm the existence of aliphatic, alkene, aromatic, keto, and ester functionalities in lime cutin (Zlotnik-Mazori and Stark, 1988). It is also possible to distinguish between rigid and mobile carbons using cross polarization with dipolar dephasing. A combination of spin-lattice relaxation times (^{13}C T_1), rotating-frame ^1H relaxation times (^1H $T_{1\rho}$), and line width measurements show that bulk methylenes and primary alcohol esters have a flexible character, while secondary alcohol esters are found at cross-link sites and show motional constraints.

The recently developed technique of high-resolution magic angle spinning (HR-MAS) NMR spectroscopy allows for the analysis of materials that swell, become partially soluble, or form true solutions in a solvent even when some solids are still present (Keifer et al., 1996; Millis et al., 1997). Stark et al. (2000) and Fang et al. (2001) used heterocorrelated multiple-quantum coherence (HMQC) NMR spectroscopy for comparing the structure of intact lime cutin swelled in DMSO, with that of an oligomer (obtained by selective hydrolysis of hindered esters using iodotrimethylsilane) dissolved in $CHCl_3$. Although the spectra confirm the presence of CH_2O and CH_2 groups, detailed assignments of all signals were not made. The quantitative nature of the technique was confirmed by comparing spectra from dry and wet cutin (Stark et al., 2000). CPMAS NMR spectroscopy has been used to identify the major carbon functionalities present in intact biopolymers, but two-

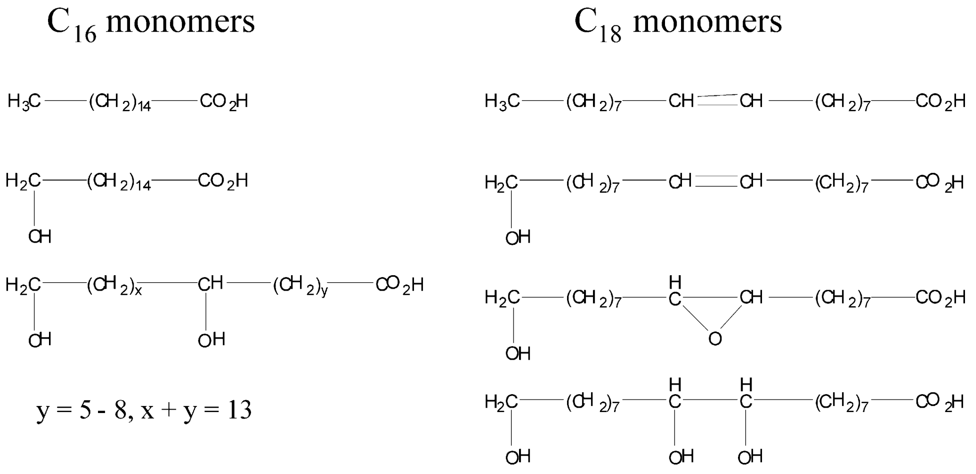


Fig. 1. Cutin monomers identified from previous depolymerization studies (adapted from Kolattukudy (1984))

dimensional techniques are essential for resolution of overlapping signals.

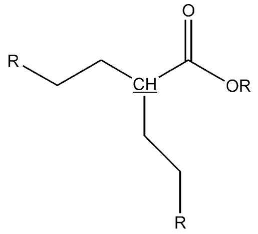
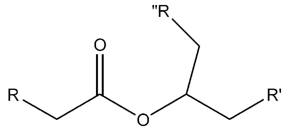
We report here a one- and two-dimensional HR-MAS NMR study coupled with a one-dimensional CPMAS and Bloch decay study for characterization of the molecular architecture of cutin isolated from tomato fruit cuticle, with possible evidence for presence of cross-linking. One-dimensional ^{1}H HR-MAS, two-dimensional TOtal Correlation SpectroscopY (TOCSY), and Heteronuclear Single Quantum Coherence (HSQC) are performed on tomato cutin swollen in DMSO. Detailed through-bond connectivities and assignments are made based on the data obtained. To our knowledge, this is the first detailed account of HR-MAS NMR spectroscopic structural analysis of tomato cutin.

2. Results and discussion

^{13}C CPMAS solid-state NMR spectroscopy gives lines that are broader than those observed for materials in solution, mainly from the chemical shift anisotropy and the lack of molecular motion. After swelling in DMSO, materials have enhanced molecular mobility, and magic angle spinning aids in the removal or minimization of chemical shift anisotropy, dipole-dipole interactions and magnetic susceptibility line broadening (Keifer et al., 1996; Millis et al., 1997; Stark et al., 2000; Fang et al., 2001). Using two-dimensional techniques, we identify carbons and protons that fall into groups on the basis of their structures as A-type (terminal methyls and main-chain methylenes), B-type (methylenes attached to the oxygen (O)-side of an ester), C-type (methylenes attached to the carbonyl (C=O) side of an ester), D-type (methylenes in free primary alcohols), E-type (methines and methylenes in free secondary alcohols), F-type (methylenes in free fatty acids), G-type (methines in α-branched carboxylic acids/esters), and H-type (methines attached to ester-linked mid-chain hydroxyls), (Table 1).

Total correlation spectroscopy (TOCSY) identifies through bond couplings between protons in the same

Table 1
Types of carbons and protons with chemical shifts

Symbol	Type	^{1}H shift (ppm)	^{13}C shift (ppm)
A_1	$\underline{CH_3}$-CH_2-CH_2-CH_2-R	0.8	14
A_2	CH_3-CH_2-$(\underline{CH_2})_n$-CH_2-R	1.2	30
B_1	R-CH_2-CH_2-CH_2-CO-O-$\underline{CH_2}$-CH_2-CH_2-R	4.0	66
B_2	R-CH_2-CH_2-CH_2-CO-O-CH_2-$\underline{CH_2}$-CH_2-R	1.6	30
B_3	R-CH_2-CH_2-CH_2-CO-O-CH_2-CH_2-$\underline{CH_2}$-R	1.2	26
C_1	R-CH_2-CH_2-$\underline{CH_2}$-CO-O-CH_2-CH_2-CH_2-R	2.2	34
C_2	R-CH_2-$\underline{CH_2}$-CH_2-CO-O-CH_2-CH_2-CH_2-R	1.5	22
D_1	R-CH_2-CH_2-$\underline{CH_2}$-OH	3.4	62
D_2	R-CH_2-$\underline{CH_2}$-CH_2-OH	1.4	34
D_3	R-$\underline{CH_2}$-CH_2-CH_2-OH	1.2	26
E_1	R-CH_2-CH_2-$\underline{CH}$OH-CH_2-CH_2-R	3.4	71
E_2	R-CH_2-$\underline{CH_2}$-CHOH-$\underline{CH_2}$-CH_2-R	1.3	38
E_3	R-$\underline{CH_2}$-CH_2-CHOH-CH_2-$\underline{CH_2}$-R	1.2	26
F_1	R-CH_2-$\underline{CH_2}$-CO_2H	2.2	34
F_2	R-$\underline{CH_2}$-CH_2-CO_2H	1.5	26
G		2.4	44
H		4.8	74
L	R-CH_2-$\underline{CH}$=CH-CH_2-CH=CH-R	5.4	132

spin system. Fig. 2 shows the HR-MAS TOCSY spectrum for tomato cutin. The chemical shifts of the interacting protons are obtained by drawing lines: perpendicular to the horizontal and vertical axes. A cross-peak describes, in one dimension, the chemical shift of a particular set of protons, and in the other, the chemical shift of the protons to which they are coupled. For example, the TOCSY spectrum for tomato cutin shows a cross-peak at 4.0 ppm (B), which is from protons on methylenes (CH_2) directly attached to the singly bonded oxygen (O) atoms of esters. These protons are coupled to protons of chemical shift 1.6 ppm (CH_2 β to the O), and 1.2 ppm (CH_2 γ to the O). The cross-peak at 2.2 ppm is from protons on CH_2s that are directly attached to carbonyls (C=O) in ester or carboxylic acid functional groups (C or F-type). These CH_2s are coupled to protons at 1.5 ppm (CH_2 β to C=O) and 1.2 ppm (CH_2 γ to C=O). This, in addition to the HSQC cross-peaks (shown later), is evidence for presence of ester type functionalities in cutin.

A feature not identified in tomato cutin through previous depolymerization studies is the presence of α-branched carboxylic acids and esters having the structure G depicted in Table 1. The TOCSY spectrum exhibits a cross-peak at 2.4 ppm (G) in addition to the one at 2.2 ppm from straight chain fatty acids/esters (C/F). This peak at 2.4 ppm is from methines that are attached to carboxyl groups in α-branched carboxylic acids/esters. Such methines have an alkyl substituent, in addition to the carboxyl group. The methine protons couple with peaks at 1.5 ppm (β CH_2) and 1.2 ppm (γ CH_2). There is further confirmation for this peak from the cross-peak seen in the HSQC spectrum of tomato cutin, which shows the chemical shift of attached carbons as being 44 ppm (shown later), which correlates well with the reported ^{13}C chemical shifts for α-branched carboxylic esters (Dostolova et al., 1983). Such a peak at 2.4 ppm, aside from the peak at 2.2 ppm from straight chain fatty acids, has also been observed in ^1H NMR spectra of lime cutin by Fang et al. (2001) who did not make such a specific assignment. Branched fatty acids have been detected in trichome-exudated sugar esters in certain *Solanaceae* species, and it is believed that they are formed by a biosynthetic pathway that is different from that of straight chain fatty acids (Kroumova et al., 1994). Esters of branched fatty acids are known to require harsher saponification conditions as compared to esters of straight chain fatty acids (Fujii et al., 1986). It is expected that this difficulty would be exacerbated in esters of α-branched fatty acids, due to steric effects. It is possible that under the depolymerization conditions used, most of the branched fatty acids remain as components of oligomers and are therefore undetected by gas chromatography. Osman et al. (1995) carried out aqueous base hydrolysis of tomato cutin. Aside from a dimer of 10,16-dihydroxyhexadecanoic acid, minor quantities of other dimers of dihydroxyhexadecanoic acids were also obtained, which the authors believe could also contain branched fatty acids. Similarly, Baker and Holloway (1970) hydrolyzed tomato cutin with ethanolic KOH and obtained 10,16-dihydroxyhexadecanoic acid (71%) and 16-hydroxyhexadecanoic acid (5%) as the main monomers along with some unidentified acids. These could be branched carboxylic acids. This feature of tomato cutin monomers has not been recognized in the studies to date.

Tomato cutin is also seen to have mid-chain (secondary) alcohols as indicated by the peak at 3.4 ppm from methine (CH) protons attached to hydroxyl groups (E-type). These protons couple to other protons at 1.3 ppm (β to OH) and 1.2 ppm (γ to OH). The cross-peak at 4.8 ppm is believed to be from methine protons (CH) that are directly attached to mid-chain hydroxyl groups involved in ester linkages (H-type). This assignment is based on chemical shifts in known compounds (SDBSWeb). The cross-peak at 5.4 ppm is from protons on olefinic carbons (L-type). The data also points to aliphatic chains with two double bonds separated by a CH_2 unit as seen from the coupling with protons at 2.7 ppm, and 2.0 ppm (α to double bond on the other side). There are cross-peaks in the aromatic region (6.0–7.5 ppm), but the structural information is limited and assignments cannot be made. From the weak intensities of the aromatic protons in the 1-D spectra, they appear to be minor components. Since the aromatic signals appear rather sharp in the TOCSY, we believe that they could be from smaller well-solubilized aromatic molecules that were co-extracted during isolation. The cross-peaks from the biopolymers themselves will be broad and weak due to faster relaxation.

Heteronuclear single quantum coherence (HSQC) spectroscopy correlates the proton chemical shift with

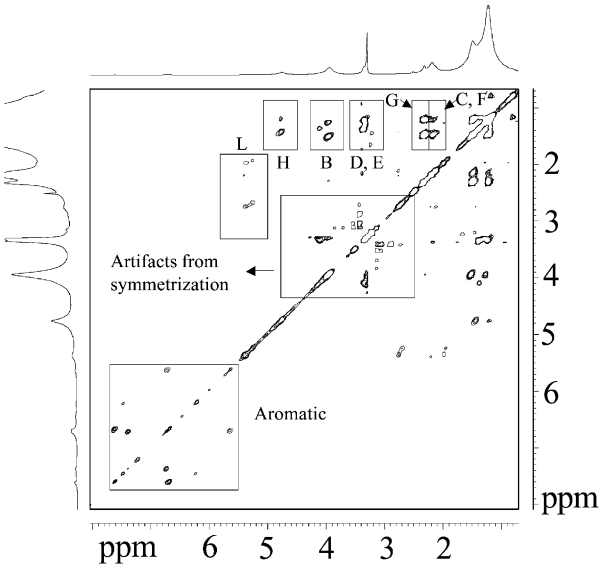


Fig. 2. 600 MHz ^1H–^1H HR-MAS NMR TOCSY spectrum of tomato cutin swollen in DMSO-d_6 while spinning at 9kHz.

that of the carbon to which it is directly attached. A cross-peak describes in one dimension the proton chemical shift of a C-H unit, and in the other, the chemical shift of the attached carbon. The cross-peaks that have been identified in the spectrum (Fig. 3) have been labeled by symbols whose assignments are listed in Table 1. Cross-peak B_1 results from CH_2s directly attached to singly bonded ester O atoms and B_2 from CH_2s β to the O atom. The γ carbon atoms and associated protons (B_3) are assigned by deduction as part of a large cross-peak that includes other species. The cross-peak labeled C_1 is consistent with CH_2s α to C=O of esters, and also the same units in fatty acids (F_1). However the units β to the C=O group in fatty acids or esters, display two signals with a proton shift of 1.5 ppm and carbon shifts of 22 and 26 ppm. Differentiation of these two peaks from the small chemical shift differences in the HSQC is difficult using conventional NMR databases. These peaks are seen in the *Agave americana* leaf cuticle (which has both cutin and cutan), however the cross-peak (1.5, 22) is lost after the saponification reaction that cleaves esters (data not shown). We believe that the signals in the box labeled 'C_2F_2' have significant contribution from esters as well as free acids, with the peak at a ^{13}C shift of 22 ppm that is lost upon saponification assigned to esters. These ester, acid, and alcohol units are in polymethylenic chains in which the main chain methylene and terminal methyl groups are described by cross-peaks A_2 and A_1, respectively.

In addition to esters, terminal and mid-chain alcohols can also be identified. Carbons and associated protons in a primary alcohol chain are described by D_1 (CH_2s α to OH), D_2 (β -CH_2s), and D_3 (γ-CH_2s, this cross-peak falls under a crowded region that contains cross-peaks from other aliphatic moieties). Mid-chain alcohols are described by cross-peaks E_1 (3.4, 71) assigned to CH_2s α to OH, the β-CH_2s E_2 (1.3, 38), and the γ CH_2s E_3 (1.2, 26).

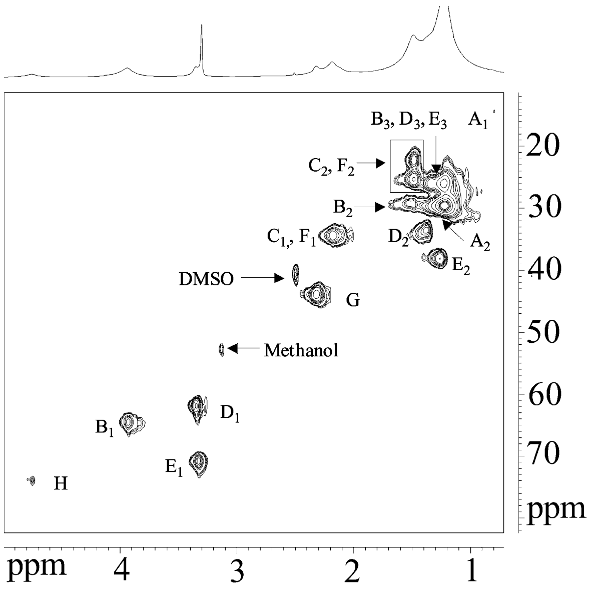


Fig. 3. 600 MHz ^{1}H–^{13}H HR-MAS NMR HSQC spectrum of tomato cutin swollen in DMSO-d_6 while spinning at 9 kHz.

The peak labeled G (2.4, 44) is a strong cross-peak consistent with methines α to C=O groups in α-branched carboxylic acids or esters. Such α-branched carboxylic acids have not been identified in any of the degradative studies involving tomato cutin. Such units are expected in a cross-linked esterified biopolymer. Dostolova et al. (1983) have reported ^{13}C chemical shifts of α-substituted long chain carboxylic acids and esters, which correlate well with the values observed for G. Units β and γ to the functionality in such structures are difficult to distinguish because they coincide with similar units in straight chain esters (C) and fatty acids (F). We can conclude from other studies that such α-branched carboxylic acids are esterified in *Agave* cutin, since the cross-peak is entirely lost during saponification to cutan (data not shown).

Fang et al. (2001) used gradient assisted HMQC NMR spectroscopy to identify directly bonded carbon-hydrogen pairs in lime cutin. They were able to identify cross-peaks from main chain methylenes, methylenes attached to the singly bonded oxygen of esters, and methylenes attached to the C=O of esters. However, they did not observe any correlations to protonated carbons resonating above 70 ppm either in the HMQC spectrum of DMSO-swelled lime cutin or in that of the soluble oligomer. Since signals in this region were observed in the ^{13}C CPMAS spectra in previous studies of lime cutin (Zlotnik-Mazori and Stark, 1988), Fang et al. (2001) believed that HR-MAS NMR spectroscopy was incapable of producing high-resolution NMR signals for its most cross-linked regions. In our study, we do observe a cross-peak H (4.8, 74) in the HSQC that is relatively weak and is consistent with structures that contain mid-chain hydroxyl groups engaged in ester formation. Structures like these are most likely responsible for cross-linking. Chemical modification of free

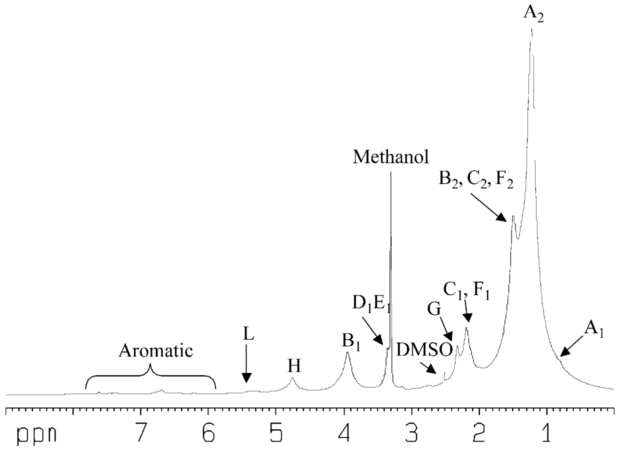


Fig. 4. 600 MHz ^{1}H HR-MAS NMR spectrum of tomato cutin swollen in DMSO-d_6 while spinning at 9 kHz.

hydroxyl groups in cutin followed by depolymerization has shown that most of the ester linkages in cutin involve primary alcohols, and the secondary alcohols are largely unesterified (Kolattukudy, 1980). Fang et al. (2001) were also able to identify terminal ethyl esters in gHMQC spectra of lime cutin [peak at (4.1, 60)]; however, no such features are observed in tomato cutin. They have also attributed a cross-peak in the intact cutin at (3.4, 61) to the α-CH_2 units directly attached to oxygens in esters ($\underline{CH_2}$O-COR), which is replaced by a cross-peak at (3.6, 63) in the oligomer ($\underline{CH_2}$OH) upon HF treatment. In our view the assignment of (3.4, 61) as above is incorrect, since such a cross-peak from α-CH_2 units directly attached to oxygen atoms in esters is in fact seen at (4, 66, B_1).

One-dimensional ^{1}H (HR-MAS) NMR and ^{13}C (CPMAS and Bloch decay) spectra (Figs. 4 and 5, respectively) show the presence of predominantly aliphatic polymethylenic units in cutin. Among the main chain CH_2 groups, the larger resonance at 29 ppm in the ^{13}C spectra has been attributed to the presence of amorphous [mixture of *gauche* and *anti* polymethylenic $(CH_2)_n$] groups, whereas the smaller shoulder at 33 ppm is from the presence of crystalline [all-*trans* $(CH_2)_n$] groups (Hu et al., 2000). While the ^{1}H spectrum of cutin (Fig. 4) has a very large aliphatic peak at 1.3 ppm from the protons of the main chain CH_2 groups (A_2), the resonance for the terminal CH_3 groups around 1 ppm is also visible. Small peaks from protons on olefinic carbon atoms are seen at 5.4 and 5.6 ppm, and those from protons on aromatic carbon atoms are seen between 6.7 and 7.6 ppm. The rest of the aliphatic ester, alcohol, and carboxylic acid signals, have been assigned on the basis of the two-dimensional spectra.

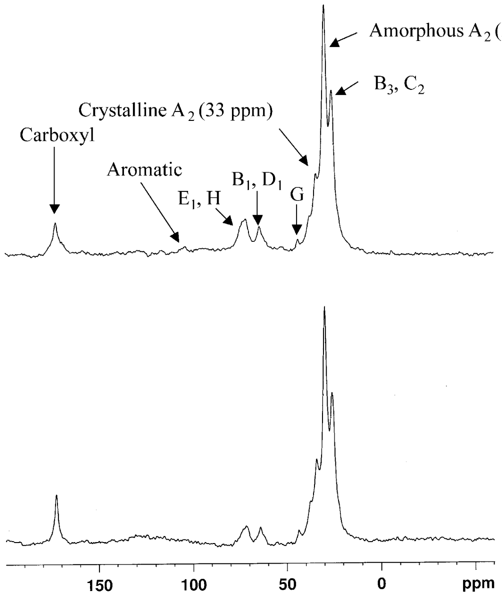


Fig. 5. (a) 300 MHz ^{13}C CPMAS NMR spectrum of tomato cutin while spinning at 13 kHz, with ramp-CP contact time of 2 ms, recycle delay of 1 s, and sweep width of 27 kHz. (b) 300 MHz ^{13}C Bloch decay NMR spectrum, while spinning at 13 kHz, recycle delay of 1 s, and sweep width of 27 kHz.

All data indicate that tomato cutin is a polyester biopolymer, consistent with findings of the degradative studies. Coupling and chemical shifts consistent with ester functionalities are seen in both the HSQC and TOCSY data. Aside from esters there is presence of primary alcohols, secondary alcohols, and free fatty acids.

There is new evidence for α-branched carboxylic acids (G) in the HSQC, although monomers with such functionalities have not been detected in the degradation studies. There is also evidence for mid-chain hydroxyls in ester linkages, however unesterified secondary hydroxyl groups are also present. We believe that the former, along with the α-branched carboxylic acids, contribute significantly to cross-linking in the biopolymer. These assignments can be confirmed using HMBC NMR spectroscopy, but the HMBC is at least two orders of magnitude less sensitive than HSQC. The H signals are weak even in the HSQC and our efforts to run the HMBC did not meet with success.

The TOCSY data confirms the presence of olefinic and aromatic carbons. The olefinic carbons (L) are part of the long aliphatic chains, and have been identified in depolymerization products. Small amount of aromaticity probably comes from the presence of *para*-coumaric acid derivatives, which have also been obtained as a product of depolymerization reactions.

Tomato cuticle has mainly cutin with ester linkages, which are hydrolyzable (Jeffree, 1996). Ether linkages are believed to exist in the non-hydrolyzable fraction of cuticles, which is called cutan, and therefore ether linkages can be excluded from tomato cutin, which is almost entirely solubilized upon base hydrolysis. Tomato cutin is not expected to have epoxide linkages because such groups have not been seen in monomers isolated through depolymerization of tomato cutin. However, such groups have been observed in depolymerization products of cutin from *Agave americana* leaf cuticle, and have also been observed by us in the HR-MAS HSQC NMR spectrum in the form of a cross peak at (2.6, 56) (data not shown).

Although glycerol has been identified as a product of depolymerization of cutins, its concentration is very low in tomato cutin (Graca et al., 2002). While unesterified glycerol shows chemical shift of (3.7, 67) and (3.4, 78) for the CH_2 and CH units, when esterified, the same units have chemical shift of (4.3, 62–66) and (5.2, 68–75), respectively. There are no visible signals in this region and it is likely that they are below the detection limit.

The possibility that DMSO is not able to access certain parts of the biopolymer cannot be ruled out, although Stark et al. (2000) have concluded that quantitative data can be collected on plant polyesters using HR-MAS NMR spectroscopy.

3. Experimental

3.1. Isolation of cutin from tomato fruit

Organically grown tomatoes were selected for isolation of cutin. The fruits were cut into 4–5 parts, and the interiors were removed. The material free of pulp and seeds was suspended in a solution of oxalic acid (0.4% w/v) and ammonium oxalate (1.6% w/v) at 40 °C for 2 days (Holloway, 1984). The cuticles came loose at this point and could be easily separated. They were washed, freeze-dried and ground in liquid nitrogen. The ground cuticles (less than 80 mesh) were successively extracted with chloroform, methanol and 1:1 chloroform/methanol (v/v) at gentle reflux for 12 h for removal of soluble lipids (Holloway, 1984). The extraction was repeated until dried extracts indicated absence of any dissolved material. The extracted residue was washed, freeze-dried and treated with a 4.5% sodium paraperiodate solution (pH adjusted to 4.1 with acetic acid) for 12 h to remove polysaccharide-like material (Zelibor et al., 1988). The filtered residue was resuspended in H_2O and refluxed for 3 h, washed, and freeze-dried to obtain cutin.

3.2. NMR spectroscopy

Solid-state CPMAS ^{13}C-NMR spectra using the ramp-cross polarization (CP) technique (Hediger et al., 1993), and two-pulse phase modulated (TPPM) decoupling (Bennett et al., 1995) technique, were obtained with a Bruker 300 MHz NMR-spectrometer (Bruker Analytic GmbH, Germany). The following optimized experimental parameters were used: ramp-CP contact time of 2 ms; recycle delay time of 1 s; sweep width of 27 kHz (368 ppm) and line broadening of 50 Hz. 90° ^{1}H and ^{13}C pulse lengths were under 5 μs. The chemical shifts were referenced to the carboxyl signal of glycine as an external secondary standard with a shift of 176.03 ppm. Freeze-dried samples were placed in a 4 mm rotor, and spun at a frequency of 13 kHz at the magic angle (54.7° to the magnetic field). Contact time of 2 ms was determined to be optimum for all types of carbon functionalities. The Bloch decay (BD) MAS ^{13}C NMR spectra were acquired using an 18°-pulse angle and a recycle delay of 2 s.

HR-MAS NMR experiments were carried out on a Bruker Avance 600 MHz DRX, fitted with a ^{1}H, ^{13}C, ^{15}N HR-MAS probe. Sample (~30 mg) was swollen in DMSO-d_6 and packed into a 4 mm Ziconia Rotor with a Kel-F cap. All spectra were obtained using 9 kHz spinning speed. Proton spectra were collected with composite pulse presaturation using 256 scans, 25 ppm sweep width, and 2 s recycle delay. TOCSY {256 scans, TD (F_1) 1024, TD (F_2)} were acquired using time proportional phase incrementation (TPPI), and a mixing time of 50 ms. HSQC (196 scans, TD (F_1) 1024, TD (F_2) 512, J^1 (^{1}H–^{13}C) 145 Hz) were acquired using sensitivity enhancement and gradients for coherence selection with decoupling during acquisition. All the 2-D data sets were processed using sine-squared functions with phase shifts of 90° in both dimensions.

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