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ARTICLES

Using Trifluoroacetic Acid To Augment Studies of Potato Suberin Molecular Structure

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Systematically varied reaction times and concentrations of trifluoroacetic acid (TFA) have been used to remove polysaccharides associated with suberin isolated from potato wound periderm, thereby augmenting spectroscopic determinations of the molecular structure of this protective plant polyester. Treatments with dilute TFA left a residual insoluble material for which both solid-state ^{13}C and ^{1}H NMR spectra displayed significant improvements in resolution without compromising the integrity of the protective plant polyester, whereas higher concentrations of TFA made it possible to achieve controlled hydrolysis of the suberin aliphatic or aromatic domains. Among the isolated fragments were two hydroxyphenyl derivatives reported previously in lignins and a novel aliphatic-aromatic ester trimer that is identified provisionally. Together these protocols help to characterize the carbohydrate types that are bound covalently to the suberin polyester and to identify the interunit covalent linkages among the aliphatic ester, phenolic, and carbohydrate moieties in suberized potato tissue. The strategies described herein may also advance molecular-level investigations of lignocellulosic materials or vegetable tissues that exhibit strengthened intercellular adhesion.

KEYWORDS: Suberin; *Solanum tuberosum*; potato; trifluoroacetic acid; CPMAS; HRMAS; NMR

INTRODUCTION

Suberin is an essential plant biopolymer that is typically found within underground organs and periderms and is also synthesized after wounding to protect tissues from moisture loss and to reduce their susceptibility to bacterial and fungal attack (*1*, *2*). Whereas it has been recognized for many years that suberized cells contain both poly(aliphatic) and poly(phenolic) domains (*3*, *4*), their detailed structures and molecular connectivities remain incompletely delineated, in part because the polymer occurs as an integral unit together with cell-wall polysaccharides and cannot be extracted into aqueous or organic solvents (*5*). Enzymatic and extractive protocols (*4*, *6*) have been optimized to remove ~95% of the unsuberized cell walls and waxes, respectively, from suberized potato periderm, facilitating the use of methods such as solid-state ^{13}C NMR to identify structural types directly within the intact biopolymer (*4*−*6*). Nevertheless, the remaining cell-wall polysaccharides, which are likely to be covalently attached to the polyester and thus inaccessible to enzymatic attack (*7*), can obscure structurally informative information that could otherwise be deduced for suberin from the NMR spectra or other spectroscopic data.

Among the methods available to hydrolyze the cell-wall polymers to monosaccharides, the most common involve the use of concentrated mineral acids (HCl, H_2SO_4), although such acids also promote unwanted side reactions (*8*). Particularly for non-cellulosic polysaccharides, hydrolysis is also possible with trifluoroacetic acid (TFA), with the added advantage that its volatility allows for removal by evaporation or lyophilization rather than a loss-prone neutralization step (*8*, *9*). Depending on the aqueous concentration that is employed, TFA may serve as a solvent or hydrolytic reagent for cellulose and ligninocellulosic materials (*8*−*12*).

In the current work, a systematic study of TFA concentrations and reaction times has been made to optimize removal of residual cell-wall polysaccharides from suberized potato wound periderm in which cell-wall materials had been hydrolyzed previously by standard treatments with cellulase and pectinase (*7*). This newly developed procedure has two important benefits for the investigation of suberin molecular structure. First, treatments with dilute TFA (0.5 M for 30 min or 0.1 M for 60 min) produce significant improvements in resolution for both magic-angle spinning ^{13}C and ^{1}H NMR spectra, which by comparison with published spectra of wood (*13*) allow us to provisionally identify β (1−4)-D-glucopyranose (cellulose) and β (1−4)-D-xylopyranose (hemicellulose) structures that may be

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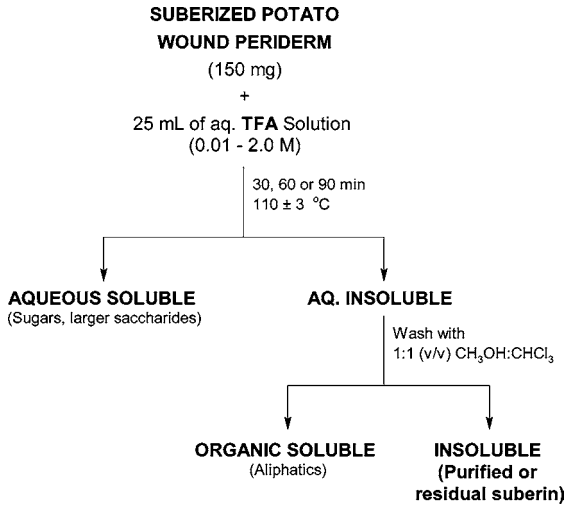
Scheme 1. Treatment of Suberized Potato Tissue with Trifluoroacetic Acid (TFA)



covalently bound to the suberin polyester. Second, controlled hydrolysis of the suberin aliphatic domain is possible using TFA at concentrations exceeding 1.0 M, yielding soluble products that may retain interunit linkages among the aliphatic ester, phenolic, and carbohydrate moieties in this potent but complex protective plant biopolymer. Among the products is a novel aliphatic-aromatic ester oligomer that has been identified provisionally. Such molecular information is essential to permit rational design of crop protection strategies focusing on the attack of microbial pathogens, and such chemical treatments could be applicable to related lignocellulosic materials or potato tissues that exhibit strengthened intercellular adhesion.

MATERIALS AND METHODS

Chemicals. Trifluoroacetic acid and tissue culture water were purchased from Sigma-Aldrich (St. Louis, MO). The enzymes *Aspergillus niger* cellulase (EC 3.2.1.4) and *A. niger* pectinase (EC 3.2.1.15) were purchased from ICN Biomedicals (Aurora, OH) and Sigma Chemicals, respectively. Other laboratory chemicals were all of reagent grade or better.

Preparation of Potato Suberin. Suberization of wounded potatoes (*Solanum tuberosum* L. cv. Russet Burbank) and isolation of the biopolymer followed published procedures (*3*, *7*). Briefly, potatoes were cut into disks and suberized for 7 days in a dark aerated chamber regulated at 25 °C. The subsequent isolation procedures were as follows: blade peeling of ~1−2 mm of the periderm layer from each potato slice; thorough stirring with distilled water to remove soluble starch and residual precursor compounds; treatment with cellulase and pectinase to remove cellulose and pectin, respectively; Soxhlet extraction of the pea-sized flakes of periderm with methylene chloride (100%, 48 h) and then methylene chloride−methanol (1:1 v/v, 48 h) to remove waxes; and extraction with dioxane−H_2O (96:4 v/v) to remove residual glucose or soluble polysaccharides. The resulting solid suberized periderm was analyzed by cross polarization magic-angle spinning (CPMAS) ^{13}C NMR, confirming the similarity of our material to the potato suberin described in the literature (*5*−*7*). In addition, an aqueous-insoluble solid was released from the cellulase treatment, washed with solvents (water, methanol, and chloroform) to obtain a white material, and characterized further by CPMAS ^{13}C NMR and high-resolution MAS (HRMAS) ^{1}H NMR.

Treatment of Suberized Wound Periderm with Trifluoroacetic Acid (Scheme 1). 150 mg of the suberized material obtained as described above was stirred at 110 ± 3 °C in a stoppered flask, in separate experiments, using various concentrations of aqueous TFA (0.01, 0.1, 0.5, 1.0, and 2.0 M) and reaction times (30, 60, and 90 min). Each reaction mixture was filtered, and the insoluble material was washed with stirring using chloroform−methanol (1:1 v/v) for 2 h. The organic-insoluble material was separated by filtration, dried, and analyzed by CPMAS ^{13}C NMR. The TFA solution was coevaporated with methanol to dryness, and the resulting solids were redissolved in methanol to give a clear brown solution, which was later analyzed by solution-state NMR. To recover the aliphatic components hydrolyzed during the reaction, the 1:1 chloroform−methanol wash was also collected, rotoevaporated to dryness, and taken up in chloroform-d_3 for analysis by solution NMR. Each treatment was replicated at least twice with similar yields and spectroscopic data; subsequent exhaustive treatments yielded no additional soluble products.

Two compounds were purified from the ether extract of the aqueous-soluble fraction in **Scheme 1**, using HPLC conditions as described previously (*14*). The chloroform−methanol wash was also fractionated into products that were soluble in hexanes, chloroform, and methanol, respectively. The chloroform portion was separated into 75 3-mL fractions using Sephadex LH-20; fractions 23−27 were purified by HPTLC to give a third compound.

NMR Spectroscopy. Dry suberin residues were analyzed using standard CPMAS ^{13}C NMR experiments carried out on a Varian Instruments Unity*plus* 300 widebore spectrometer (Palo Alto, CA) equipped for solid-state NMR. The resonance frequency was 74.443 MHz, with a customary acquisition time of 30 ms, a delay time of 2 s between successive acquisitions, and a CP contact time of 1.5 ms. Typically, each 30-mg sample was packed into a 5-mm rotor and supersonic MAS probe from Doty Scientific (Columbia, SC), then spun at 6.00 ± 0.1 kHz and room temperature for approximately 10 h. As in previous reports (*4*, *15*), no spinning sidebands were observed up- or downfield from the major carbonyl, aromatic, or aliphatic carbon peaks, presumably due to motional averaging and/or excessive broadening of such features. The resulting data were processed with 50 Hz of exponential line broadening, yielding spectra for which relative peak areas were determined by both software integration and cut-and-weigh procedures.

Soluble products and swelled suberin materials were examined using ^{1}H NMR and HRMAS ^{1}H NMR, respectively, both conducted on a Varian Instruments ^{UNITY}*INOVA* 600 spectrometer. The resonance frequency was 599.942 MHz, with a typical acquisition time of 0.684 s and a delay time of 1.5 s between successive acquisitions. The measurements were made, respectively, in a 5-mm ^{1}H-detect triple resonance probe with a pulsed z-gradient and a triple resonance nanoprobe equipped for MAS and optimized for ^{1}H detection. For HRMAS, 45 μL of DMSO was typically added to 8 mg of sample in a 4-mm glass nanotube to allow swelling in situ; MAS was conducted at 3.00 ± 0.1 kHz and room temperature without HOD suppression.

Mass Spectrometry. Electrospray and atmospheric pressure chemical ionization (ESI and APCI) were used with quadrupole detection on an Agilent Technologies (Palo Alto, CA) 1100 Series LC/MSD model G1946D mass spectrometer located at Hunter College. ESI was carried out with a drying gas temperature of 200 °C, a nebulizer pressure of 40 psi, and a flow rate of 13 L/min. APCI was conducted with a gas temperature of 300 °C, nebulizer pressure of 60 psi, and flow rate of 5 L/min. The capillary voltage was set to 4000 V, and the scanned mass range was 200−1500 amu.

RESULTS AND DISCUSSION

Five concentrations of TFA and three treatment times were assayed for the removal of residual cell-wall polysaccharides from suberized potato periderm following **Scheme 1**. As anticipated and illustrated in **Figure 1**, the largest proportions of removed materials (smallest proportions of recovered insoluble materials) were obtained with high TFA concentrations and long reaction times. For instance, the percentage of recovered material was roughly half as large with 2.0 M as compared to that with 0.01 M TFA, whereas the dependence on reaction time was modest but most significant at the highest concentrations of the acid (1.0 or 2.0 M).

Insoluble Residues. As reported previously (*6*, *15*), the CPMAS ^{13}C NMR reference spectrum of potato suberin (**Figure**

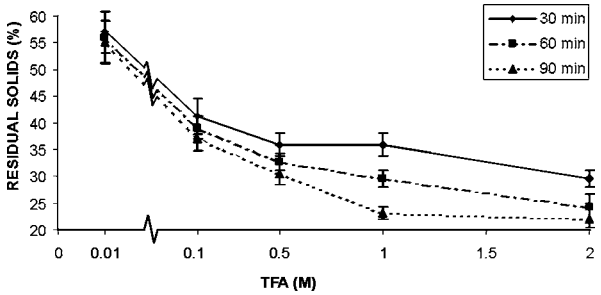


Figure 1. Yield of purified (residual solid) suberin from TFA reactions as a function of concentration and time (see **Scheme 1**), using 150 mg of starting material in each reaction. Errors estimated from duplicate reactions on nominally identical suberized periderm samples were 1–6%.

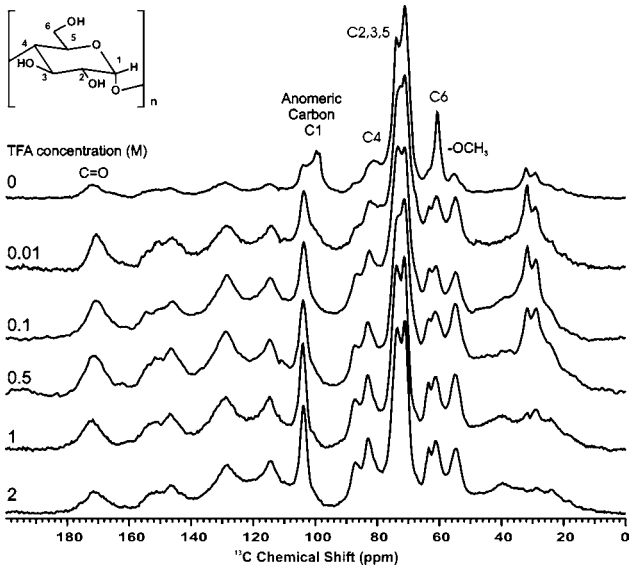


Figure 2. CPMAS ^{13}C NMR spectra of purified potato suberin after 90-min treatments with varied concentrations of TFA (as noted). No spinning sidebands were visible. Duplicate reactions yielded similar spectra.

2, top) exhibits resonances typical of aliphatic-aromatic polyesters: bulk methylenes (20−35 ppm), oxygenated aliphatic carbons (55−85 ppm), aromatics and olefinics (105−155 ppm), and carbonyl groups (170 ppm). However, the most prominent peaks belong to the carbohydrate moieties (C6 at 60 ppm, C 2,3,5 at 70−75 ppm, C4 at 83 ppm, and C1 at 101−105 ppm), some of which could overlap with oxygenated aliphatic signals from the polyester. Although the spectral features attributable to the cell-wall mixture of cellulose, pectin, and hemicellulose are narrow compared with the polyesters, the carbohydrate peaks are incompletely resolved as compared to spectra of the corresponding pure materials (data not shown).

The remaining suberin NMR spectra in **Figure 2** evidence substantial chemical changes following 90-min treatment with TFA at all of the indicated concentrations. Each of these traces shows the removal of particular polysaccharide signals: the anomeric (C1) carbon peaks at 101 and 102 ppm disappear, and the signals from C6 and C2,3,5 at 62 and 70 ppm, respectively, are clearly attenuated as compared to the reference spectrum. **Figure 3** also confirms that the signals diminished by the TFA treatment have ^{13}C chemical shifts similar to anomeric and other glycosidic carbons of water-insoluble cellulase degradation products; the resonances at 101 and 102 ppm could also be attributed to pectin (*13*). These observations suggest several possible modes of action for this acid toward the materials that remain after standard cellulase and pectinase

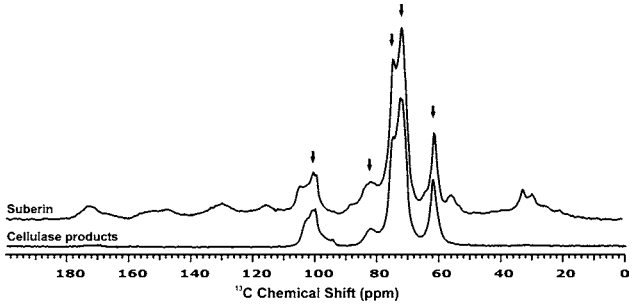


Figure 3. CPMAS ^{13}C NMR spectra of suberized potato wound periderm and insoluble cellulase degradation products. The arrows designate peaks that are attenuated or removed by TFA treatments.

enzymatic purification treatments of suberized potato wound periderm. One possibility is that moderately concentrated TFA simply solubilizes free sugars that were trapped in the suberin matrix. Given that ∼40% of a chlorogenic acid test sample is broken down by 1.0 M TFA (data not shown), the solubilization may also facilitate hydrolysis of cellulose or other polysaccharides associated tightly with the protective polyester. However, dimeric structures such as those formed by peroxidase-catalyzed reactions of sinapic acid (*14*) are found to be resistant to TFA hydrolysis (data not shown).

With the resonances at δ 62, 70, 101, and 102 ppm partially or fully removed, four well-resolved peaks flanking the large ^{13}C resonance at δ 72 ppm become evident: 83 and 89 ppm (C4 region), and 63 and 65 ppm (C6 region), as exemplified by the 90-min data in **Figure 2** (0.5 and 1.0 M traces) and also evident for 30- and 60-min durations (data not shown). If the attenuated signals signify removal of residual cellulose and/or pectin (see above), then the retained peaks could be attributed to hemicelluloses (*13*) linked to polyesters in the suberized tissue. Alternatively, these relatively sharp features in the C4 and C6 regions of the CPMAS ^{13}C spectrum could derive from unreacted crystalline cellulose at interior (65, 89 ppm) and surface (63, 83 ppm) sites (*16*−*18*).

Although peak intensities across a single CPMAS ^{13}C NMR spectrum are often unreliable quantitative indicators of the different carbon types, it is valid to monitor how the signal from a given carbon moiety varies with reaction conditions. Regardless of TFA concentration or reaction time, the polysaccharide resonance at 72 ppm, as well as the flanking peaks described above, maintain a constant intensity that indicates resistance to chemical degradation, presumably because they are involved in non-glycosidic linkages and/or located in cross-linked regions that are inaccessible to soluble reagents. Relative to the 72-ppm resonance, **Figure 4** shows that under many conditions the peak intensities for the polyester carbons (30, 33, 129, 172 ppm) remain fairly constant, but they fall off substantially at the highest TFA concentrations and longest reaction times. By contrast, the anomeric carbon resonance at 105 ppm retains or slightly increases its peak intensity at high TFA concentrations and long times. The latter trend could be attributed to preferential removal of constituents corresponding to broad spectral components, leaving crystalline cellulose or rigid glycosides linked covalently to the suberin aliphatic and/or aromatic polyester domains, either of which would cross polarize efficiently.

The peak intensity data in **Figures 2** and **4** also demonstrate the potential of these TFA treatments to effect selective hydrolysis of the long-chain aliphatic esters in suberized potato tissue. The NMR spectra show a progressive time- and concentration-dependent reduction of signal intensities at δ 30, 33, and 172 ppm ($(CH_2)_n$ and COO groups), but no effects for signals of

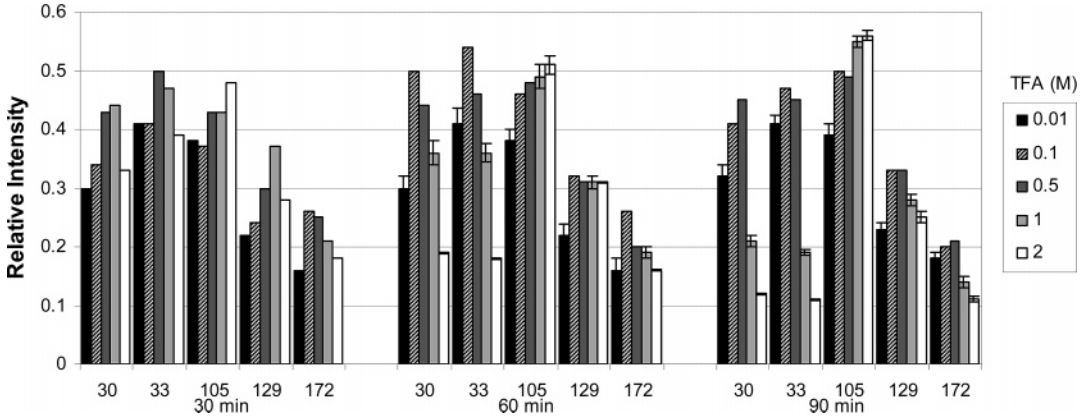


Figure 4. ^{13}C CPMAS NMR peak intensities for potato suberin hydrolyzed with different concentrations of trifluoroacetic acid (TFA) at 30, 60, and 90 min. Reported values are intensities (software-derived integrals and cut-and-weigh peak areas) relative to that of the 72 ppm polysaccharide resonance, which was verified to be invariant to the TFA treatments and normalized to 1.00 for each spectrum. Carbon types are as follows: chain methylene (30 and 33 ppm), anomeric (105 ppm), aromatic/olefinic (129 ppm), and carboxyl (172 ppm), using the assignments of Stark and Garbow (1992) (*6*). The estimated precision in peak masses from duplicate hydrolyses is 0.8–3%.

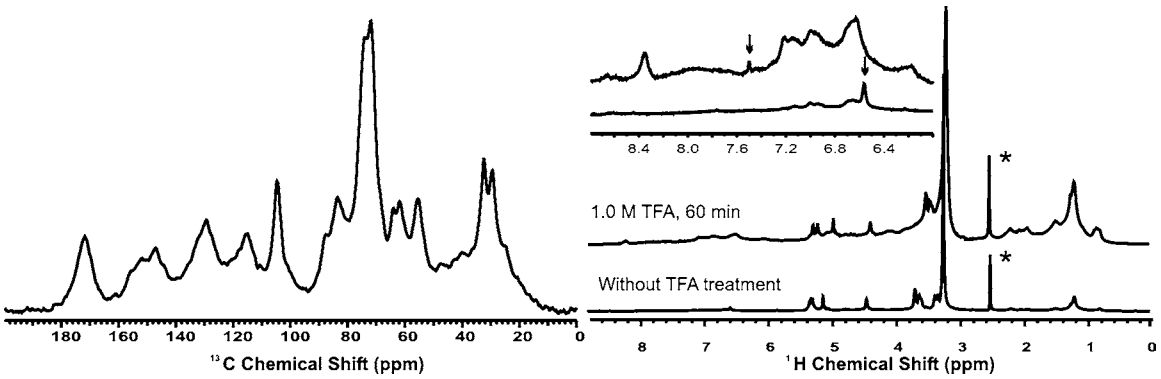


Figure 5. CPMAS ^{13}C NMR (left) and HRMAS ^{1}H NMR spectra (right) of suberin treated with 0.1 M TFA for 60 min (the arrows indicate spinning side bands; "*" shows the solvent peak). The right-hand spectra and inset include comparisons with suberized potato wound periderm before the TFA treatments.

the $-OCH_3$ (58 ppm) or aromatic moieties (115−150 ppm). For instance, with reaction times of 90 min, both the absolute and the relative intensities of these long-chain methylene and carboxyl carbon signals remain invariant using the lowest concentration of TFA (0.01 M), but they are diminished dramatically if higher concentrations of TFA (1.0 or 2.0 M) are used. A similar trend is observed at 60 min; however, under these latter reaction conditions the intensities of the aliphatic and carbonyl peaks are still preserved using 1.0 M TFA treatments, suggesting that hydrolysis of the carboxylic acids and esters present in suberin monomers, dimers, and higher oligomers is occurring but can be controlled (*2*) (W. Wang, Ph.D. Thesis, City University of NY). In fact, for a reaction time of 30 min the resulting CPMAS ^{13}C NMR spectra show no significant reductions in the peak intensities of these resonances regardless of TFA concentration.

Taken together, these observations indicate that the best conditions for enhancing the resolution of the suberin spectrum without compromising the integrity of its aliphatic domain are 0.5 M TFA for 30 min or 0.1 M for 60 min. This conclusion is reinforced by the NMR spectra displayed in **Figure 5**, which exhibit prominent resonances consistent with an aliphatic-aromatic polyester and well-resolved carbohydrate signals that no longer dominate the ^{13}C CPMAS or ^{1}H HRMAS NMR spectra of dry and solvent-swelled samples, respectively.

Soluble Products. *Water-Soluble Products.* The CPMAS ^{13}C NMR spectra in **Figure 3** also show an overall similarity between the polysaccharides retained in the enzymatically

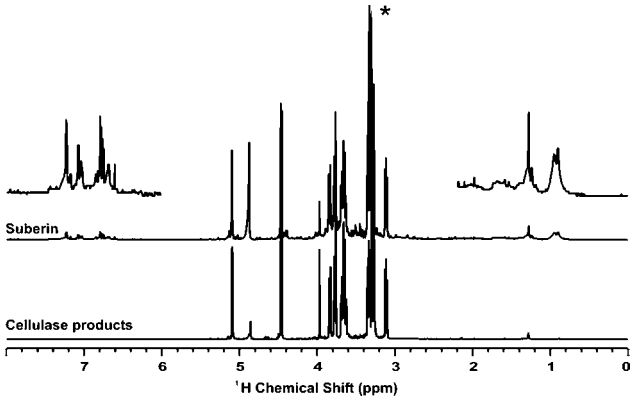


Figure 6. ^{1}H NMR spectra of aqueous-soluble products from 90-min reactions of 2.0 M TFA with potato suberin and its insoluble cellulase degradation products, respectively. The "∗" denotes a solvent peak remaining after water suppression.

treated suberized potato wound periderm and the insoluble solids released by cellulase treatment. This comparison, which has been noted earlier (*19*), is supported by the observation of comparable sugar regions (3−5 ppm) in the ^{1}H NMR spectra of water-soluble products obtained when 2.0 M TFA is used to hydrolyze both the suberized tissue and the insoluble cellulase degradation products (**Figure 6**). In contrast to these similarities between sugar-containing structures, both the suberin-derived residue from TFA treatment (**Figure 2**) and the corresponding water-

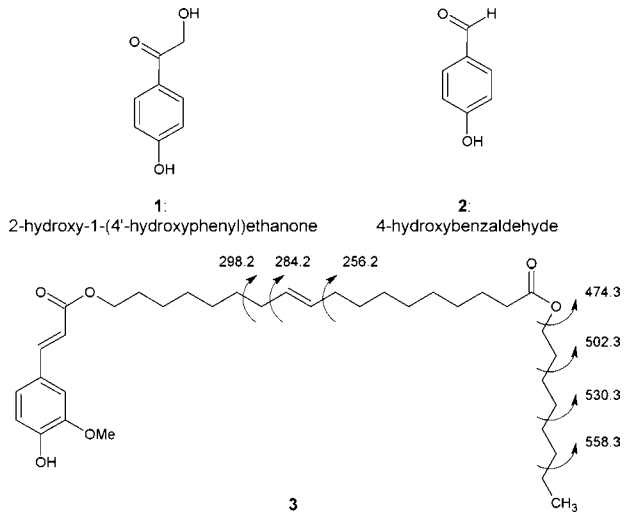


Figure 7. Compounds isolated from soluble fractions obtained after treatment of suberized potato periderm with 1.0 M TFA for 1 h. See text for experimental details.

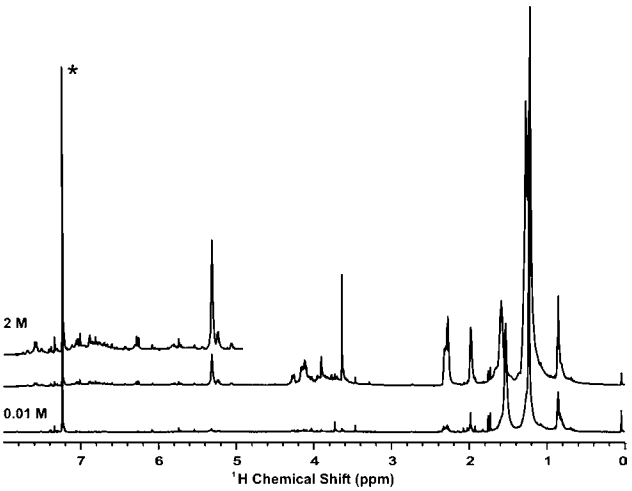


Figure 8. ^{1}H NMR spectra of product mixtures in the CH_3OH:$CHCl_3$ wash after reactions of potato suberin with 0.01 M and 2.0 M TFA, respectively. The $CDCl_3$ solvent signal serves as a reference at δ 7.23 ppm (∗). The downfield region of the 2.0 M spectrum is highlighted by an expanded inset.

soluble products (**Figure 6**, insets) uniquely display NMR resonances from aromatic and aliphatic moieties, consistent with the hypothesis that the polyester is bound covalently to the sugar structures (*4*). An ethyl ether extract of these products yielded 2-hydroxy-1-(4′-hydroxyphenyl)ethanone (**1**) and 4-hydroxybenzaldehyde (**2**) (**Figure 7**), compounds reported previously in lignin (*20*). Additional studies are underway to elucidate the specific linkages among aromatic, aliphatic, and polysaccharide moieties.

Organic-Soluble Products. Although the major products of the TFA reactions are sugar-containing materials, the most vigorous conditions (1.0 M, 90 min or 2.0 M, ≥60 min) yield significant amounts of aliphatic suberin fragments that are soluble in chloroform−methanol mixtures. The removal of aliphatics by TFA, demonstrated above in **Figures 2** and **4**, yields organic-soluble products with the ^{1}H NMR spectra displayed in **Figure 8**. The observed ^{1}H chemical shifts indicate the presence of long-chain aliphatic compounds (δ 0.9 ppm, $-CH_3$; 1−1.8 ppm, $(CH_2)_n$), probably dominated by unsaturated acids (δ 2.0 ppm, $-CH_2-C=$; 2.2−2.4 ppm, $-CH_2-C=O$; 3.6 ppm, $-OCH_3$; and 5.2−5.4 ppm, $-CH=CH-$) and including small portions of long-chain alkanols and/or glycerol (δ 3.6 ppm, $-CH_2OH$) and aromatics (δ 6.2−7.6 ppm, mainly hydroxycinnamic acids). These findings are reasonable in light of prior reports that the major monomeric constituents in the aliphatic domain of suberin are long-chain α,ω-diacids, ω-hydroxyacids, alkanoic acids, and *n*-alkanols (*21*, *22*). Moreover, the presence of a group of NMR signals at δ 3.2−4.4 ppm suggests possible esterification of these compounds through their acid and hydroxyl groups to form dimers, trimers, and higher oligomers, or the presence of glycerol- and hydroxycinnamic acid-linked polyesters.

This hypothesis is supported by structural analysis of a compound isolated from this extract, which indicates the presence of a hydroxycinnamic acid linked to an aliphatic long-chain compound. The ^{1}H NMR spectrum shows two olefinic protons trans to one another (H_α, 7.6 ppm and H_β, δ 6.25 ppm, $J = 15.8$ Hz) and linked by a through-bond HMBC connectivity to an aromatic system (δ 6.9−7.12 ppm) with a methoxy group (δ 3.8 ppm), as well as a carboxylic group (δ 167.8 ppm) similarly connected to H_α (δ 7.6 ppm) in the gHMBC spectrum, that is, the presence of a ferulic acid structure.

This carboxylic group also shows HMBC connectivity with other protons (δ 4.19 ppm), suggesting a ω-hydroxyacid unit substituted on an ester carbonyl group (**Figure 7**, **3**). In turn, these latter protons show further interactions with methylene groups at δ 25.9 and 28.1 ppm, indicating the presence of a long-chain aliphatic compound. The carbonyl group (δ 168.4 ppm) of the ω-hydroxyacid is coupled with protons at δ 2.22 ($-OCO-CH_2-$) and 4.12 ppm ($-CH_2-O-CO-$), indicating the presence of another esterified aliphatic chain. Finally, the elucidation of **3** as the major component of this extract is supported by MS data: both ESI (negative mode) and APCI (negative mode) MS of this fraction show a molecular ion at *m*/*z* 585.4 [M − H] as well as fragments at 558.3, 530.3, 502.3, 474.3, 298.2, 284.2, and 256.2. Additional work is in progress to confirm the identification of this and other compounds.

Taken together, the results presented above show that an efficient and reproducible method has been developed to remove non-cellulosic polysaccharides that remain after exhaustive enzymatic methods of wound-healing potato suberin isolation. The availability of this protocol enables us to augment structural studies of this protective plant polyester and could be more broadly applicable to molecular-level studies of lignocellulosic materials or vegetable tissues that exhibit strengthened intercellular adhesion. When the goal is to obtain the best possible resolution of suberin structural features and connectivities without compromising its integrity, then 0.5 M TFA for 30 min or 0.1 M TFA for 60 min yield the best results, but the amounts obtained in these reactions are less than 50% of the original enzymatically treated preparations. A lower concentration of TFA (0.01 M) retains about 60% of the initial material and removes only sugars, but the enhancement of the aliphatic and aromatic NMR signals is suboptimal. When the goal is to produce soluble fragments that reveal covalent connectivities between the suberin polyester and its associated polysaccharide cell walls, then the appropriate reaction conditions are more drastic: perhaps 2 M TFA for 60 min. In addition to the soluble products identified in the current study, work is underway to elucidate the full variety of aliphatic, sugar-aliphatic, and aromatic-aliphatic linkages that typify the suberin structure and could be targeted in efforts to design more robust protective strategies against microbial invasion of plant tissues.

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