Isolation and Spectral Characterization of Plant-Cuticle Polyesters

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An efficient protocol combining enzymatic and chemical methods has been developed for the isolation of lime fruit cutin and potato suberin in pure form and with excellent yields. Starting with the procedures specified by commercial assay kits for model substrates, the breakdown of cellulose, pectin, and hemicellulose cell-wall constituents was optimized with respect to enzyme concentration, substrate concentration, reaction time, and temperature. The optimized procedures were then applied to the plant polyesters cutin and suberin, with the isolation process monitored in parallel by gravimetric methods and with cross-polarization magic-angle spinning ^{13}C NMR. For lime cutin, the optimized protocol avoids spectral interferences from cell-wall carbohydrates, waxes, and exogenous oxalate, confirming that esters of both primary and secondary alcohols are present in the biopolymer. For suberized potato periderm, the optimized protocol removes more than 95% of the unsuberized cell walls and waxes, making it possible to generate an NMR difference spectrum of suberin that includes resonances from all major functional groups of this aromatic–aliphatic polyester.

INTRODUCTION

Cutin and suberin are the structural polymers of plant cuticle, a material that regulates interactions between plants and their environment (Holloway, 1982; Kolattukudy, 1984). To develop an understanding of cuticular protective functions at the molecular level, it is important to learn how the monomeric units of these biopolymers are linked together and bonded within wax and cell-wall matrices. Spectroscopic approaches to these questions have been hampered by the insolubility of the plant materials in both aqueous and organic solvents.

Recently, we and others have used magic-angle spinning ^{13}C nuclear magnetic resonance spectroscopy (MAS ^{13}C NMR) to study the structure and dynamics of intact cutin, suberin, and lignin (Maciel et al., 1985; Lewis et al., 1987; Irwin, 1989; Zlotnik-Mazori and Stark, 1988; Stark et al., 1989; Garbow et al., 1989). In addition to providing estimates for the numbers and types of functional groups present in various tissue samples, ^{13}C NMR spectra and spin–relaxation experiments have supplied new information about cuticle flexibility and cross-linking (Zlotnik-Mazori and Stark, 1988), hydrophobic association between cutin and wax constituents in lime cuticle (Garbow and Stark, 1990), and attachment of suberin to carbohydrates in the potato wound periderm (Stark and Garbow, 1992).

Published protocols for the isolation of cutin and suberin (Walton and Kolattukudy, 1972; Deas et al., 1974; Kolattukudy and Dean, 1974; Schonherr, 1979; Holloway, 1982) are convenient and have proven to be quite satisfactory for the MAS NMR studies. However, in our hands, they lacked the reproducibility required to yield meaningful ^{13}C NMR difference spectra of cuticular materials obtained before and after partial depolymerization, with varying suberization conditions, or with the incorporation of site-specific ^{13}C labels. Moreover, we sought a greater isolation efficiency for large-scale cutin preparations used in subsequent depolymerization experiments and in experiments monitoring the time course of suberization (R. E. Stark, W. Sohn, R. A. Pacchiano, Jr., M. Al-Bashir, and J. R. Garbow, unpublished results).

For these reasons, the enzymatic treatments for cell-wall removal were optimized using model substrates. We started with the experimental parameters recommended in commercial assay kits for cellulase, pectinase, and hemicellulase. In each case, enzyme and substrate concentrations, reaction time, and temperature were varied systematically to maximize the yield of hydrolyzed product. The optimized protocols were then applied to lime cuticle and suberized potato tissue, and their efficacy was judged by both gravimetric and spectroscopic criteria. Finally, these isolations of plant-cuticle polyesters were compared to previously published procedures.

MATERIALS AND METHODS

Enzymatic Hydrolysis of Cell-Wall Model Substrates. Except as noted, all reagents were obtained from Sigma Chemical Co. (St. Louis, MO). Following the standard assay protocol provided by Sigma, purified cellulose at a concentration of 50 mg/mL was suspended in 50 mM sodium acetate buffer (pH 5) at 37 °C and treated for 2 h with *Aspergillus niger* cellulase (EC 3.2.1.4), present at 0.4–1.2 mg/mL (5.1 units/mg). The reaction was carried out in a thermostatically controlled incubator shaker (New Brunswick Instruments, New Brunswick, NJ), using a stir bar for mechanical agitation. After the reaction was quenched in ice, the concentration of glucose products was measured by phosphorylation and reaction with nicotinamide adenine dinucleotide (NAD) to produce NADH; the latter product was determined spectrophotometrically by its absorbance at 340 nm. Each hydrolysis experiment was checked against a blank containing no enzyme. Modifications to the concentrations, time, and temperature are described under Results.

Polygalacturonic acid from oranges was suspended at 5 mg/mL in 50 mM acetate buffer (pH 4) at 25 °C and treated for 5 min with *A. niger* pectinase (EC 3.2.1.15), present at 11 mg/mL (9.0 units/mg). The reaction was run with mechanical agitation in a mechanical shaker or thermostated incubator shaker and quenched with a 0.1 M I_2/0.2 M KI/1.0 M Na_2CO_3 solution. The concentration of galacturonic acid products was measured by iodometric titration with thiosulfate and referenced to a blank reaction run without enzyme present (Kolthoff and Sandell, 1952). Modifications of this standard assay are described under Results.

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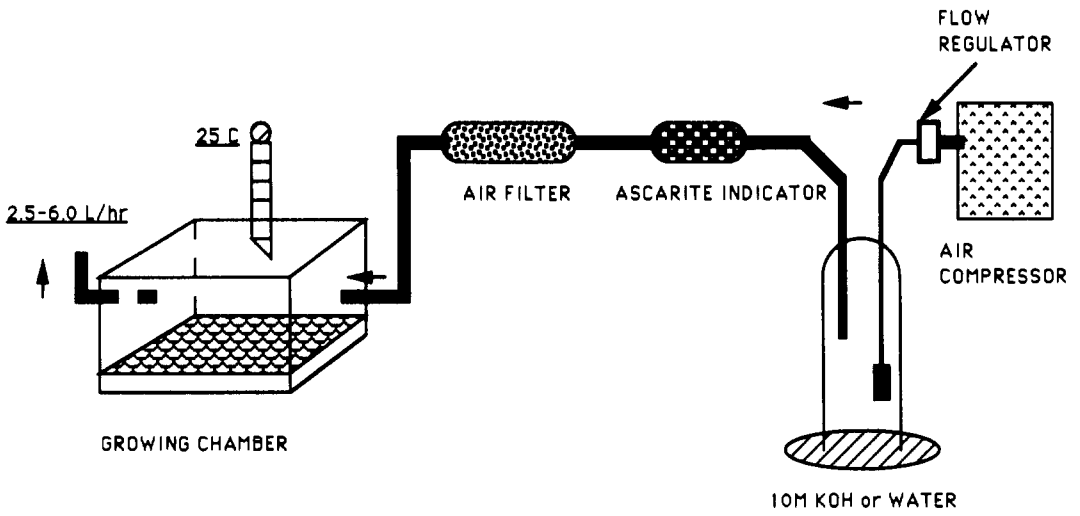

Figure 1. Growth chamber for suberization of wounded potatoes. In anticipation of future experiments using ^{13}C-labeled precursors, a KOH trap and Ascarite indicator have been added to the growth chamber to exclude and monitor CO_2, respectively.

Locust bean gum was suspended at 50 mg/mL in 50 mM acetate buffer (pH 5.5) at 37 °C and treated for 2 h with *A. niger* hemicellulase (3.9–11.8 mg/mL, 0.051 unit/mg). The reaction was carried out with mechanical agitation in a thermostatically controlled incubator shaker and quenched in ice. The concentration of galactose products was measured directly by spectroscopically monitoring the production of NADH. Modifications to the standard reaction conditions are described under Results.

Cutin from Limes. Limes (*Citrus aurantifolia*) were chosen because citrus cutin has been well studied (Holloway, 1982; Kolattukudy, 1984) and is readily available. Fruits were purchased locally and washed with distilled water; their skins were removed in large sections and soaked in distilled water for 30 min. Physical separation of the cuticle from the lime skin was carried out in two alternate ways: (a) boiling for 2 h in an ammonium oxalate buffer (16 g/L oxalate, 4 g/L oxalic acid) (Walton and Kolattukudy, 1972; Deas et al., 1974); or (b) shaking for 2–4 days at 31 °C in 4 mg/mL *A. niger* pectinase in a 50 mM acetate buffer (pH 4) (Schonherr et al., 1979).

Cuticular sheets were then treated sequentially with cellulase, pectinase, and hemicellulase enzymes derived from *A. niger*. Optimal conditions of enzyme concentration, temperature, and reaction time were determined from work with test substrates, as summarized in Table I. However, a 2.4-fold reduction of the substrate concentration was required, since the cuticular sheets absorbed solvent. Dewaxing was carried out by successive Soxhlet extractions with methanol, methylene chloride, and tetrahydrofuran (Fisher Scientific, Springfield, NJ). A typical yield of 4.5 g of dry cutin was obtained from 250 limes.

Suberin from Potatoes. Potatoes (*Solanum tuberosum* L. cv. Russet Burbank) were purchased locally and soaked briefly in a 25% Clorox solution. They were then peeled and cut into 1 × 1 × 0.4 cm sections with sterile precautions, wet with 0.30 mL of water/g of tissue, and suberized at 20 ± 3 °C for 7 days in a growing chamber through which humidified air was passed at 2.5–6.0 L/h (Kolattukudy and Dean, 1974; Garbow et al., 1989) (Figure 1).

Removal of unsuberized cell-wall materials was accomplished with between 1 and 7 cellulase treatments followed by pectinase and hemicellulase reactions, as described above. Reagent concentrations were derived from trials with test substrates, modified as shown in Table II: (a) higher concentrations and enzyme-to-substrate ratios were used when substantial cellulose was present; (b) lower concentrations, but equivalent enzyme-to-substrate ratios, were used if the tissue absorbed much of the buffer volume. Waxes were removed by successive 2-day Soxhlet extractions, as described above. Typically, 0.5 g of dry suberized potato tissue was isolated from 50 g of peeled potatoes. Unsuberized potato tissue was obtained by omitting the 7-day growth period.

Ultraviolet–Visible Spectroscopy. Absorbance of NADH at 340 nm was measured in 1-cm quartz cuvettes with a Varian DMS-300 spectrophotometer. Solutions were diluted by known factors to give absorbance readings of less than 1.5.

Table I. Optimized Conditions for Enzymatic Breakdown of Cell-Wall Model Substrates

	enzyme		
parameter	cellulase	pectinase	hemicellulase
substrate concn,[a] mg/mL	75	140	120
enzyme concn, mg/mL	5.0	6.0	80
buffer and pH	50 mM acetate, pH 5	50 mM acetate, pH 4	50 mM acetate, pH 5.5
temp, °C	44	31	45
reaction time, h	148	16	204

[a] For cutin sheets, the substrate concentrations were reduced by a factor of 2.4 for each treatment (see text).

NMR Spectroscopy. Solid-state ^{13}C NMR measurements were carried out at 27 °C on an IBM Instruments WP-200 spectrometer, operating at a ^{13}C resonance frequency of 50.33 MHz. The instrument is equipped with high-power amplifiers and a probe from Doty Scientific (Columbia, SC). Dried cutin and suberin samples, 80–300 mg in size, were ground with a Wig-L-Bug amalgamator (Spex Industries) and packed into 7-mm cylindrical, double-bearing Al_2O_3 rotors (Doty Scientific). Unless specified otherwise, spectra were acquired with 5.0-kHz magic-angle spinning using matched 50-kHz ^{13}C–^{1}H polarization transfers of 0.4–1.5 ms, 50-kHz ^{1}H decoupling fields, and 1-s recycle delays. Chemical shifts are quoted with respect to TMS using *p*-di-*tert*-butylbenzene as a secondary substitution reference.

Infrared Spectroscopy. FTIR spectral measurements were conducted at room temperature with a Spectra-Tech microscope coupled to a Nicolet 800 series FTIR spectrometer. Data were acquired using a liquid nitrogen cooled HgCdTe-A detector and a Ge on KBr substrate beam splitter. A total of 128 sample scans were collected at 4-cm^{-1} resolution using Happ–Genzel apodization.

RESULTS AND DISCUSSION

Model Substrates. Cellulase, pectinase, and hemicellulase were each allowed to react with test substrates designed to mimic plant cell walls. In a typical experiment, the yield of hydrolytic products was monitored as one of four conditions (substrate concentration, enzyme concentration, time, temperature) was varied systematically, with all others held constant. Each reaction condition was optimized with respect to the yield of products. A summary of results for the three enzyme assays appears in Table I.

Cellulase Tests: Enzyme and Substrate Concentrations. The substrate dependence of the cellulase reaction

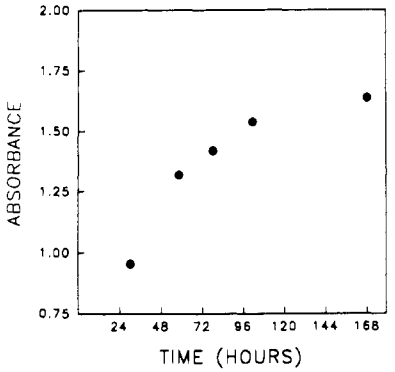

Figure 2. NADH absorbance at 340 nm from the glucose products of cellulose hydrolysis, obtained as a function of reaction time at 37 °C with 75 mg/mL cellulose, 1.0 mg/mL cellulase, and pH 5.

was studied in a series of 22-h reactions at 37 °C, conducted with an enzyme concentration of 1.0 mg/mL. Cellulose substrate concentrations were varied from 20 to 75 mg/mL. The largest yield of glucose was achieved with a cellulose concentration of 75 mg/mL, suggesting that substrate concentrations at least this high should be employed for cell-wall removal from lime cutin and potato suberin. (Higher substrate concentrations gave a thick slurry.) To determine the cellulase dependence of this reaction, enzyme concentrations were varied between 1 and 7 mg/mL in reactions run for 7 days at 37 °C with a cellulose concentration of 75 mg/mL. The maximum yield of glucose was obtained with 5.0 mg/mL cellulase.

Cellulase Tests: Time and Temperature. To determine the optimum reaction time, hydrolyses were carried out for 30–168 h at 37 °C with 75 mg/mL cellulose and 1.0 mg/mL cellulase. A shallow maximum in the yield of glucose was obtained at about 148 h (Figure 2). Finally, a series of 97-h reactions was run with 75 mg/mL cellulose and 5.0 mg/mL cellulase, at temperatures between 40 and 50 °C. The largest yield of glucose was obtained at a temperature of 44 °C. Thus, while the conditions recommended by Sigma (vide supra) are quite satisfactory for convenient assays of cellulose, achieving maximum hydrolysis of cell-wall constituents in cuticular samples calls for modifications that include substantially longer times and higher enzyme concentrations (Table I).

Pectinase Tests: Enzyme and Substrate Concentrations. The substrate dependence of the pectinase reaction was established through a series of 13-h reactions at 25 °C, carried out with an enzyme concentration of 1.1 mg/mL. Polygalacturonic acid (PGA) substrate concentrations were varied from 100 to 180 mg/mL. A shallow maximum in the amount of hydrolytic products occurred with 140 mg/mL substrate. To establish the enzyme dependence of this reaction, pectinase enzyme concentrations were varied between 1 and 10 mg/mL in reactions run at 24 °C for 18 h with a PGA concentration of 140 mg/mL. A clear maximum in galacturonic acid products was found at 6.0 mg/mL enzyme.

Pectinase Tests: Time and Temperature. Time trials of 1–25 h were conducted at 25 °C with 140 mg/mL substrate and 1.1 mg/mL enzyme. A plateau in galacturonic acid products was observed at about 16 h. Finally, the temperature dependence of pectinase hydrolysis was determined from 18-h reactions with 140 mg/mL substrate and 6 mg/mL enzyme. For reactions run between 25 and 35 °C, the maximum in hydrolytic product formation occurred at a temperature of 31 °C. All optimized parameters for the pectinase reaction are summarized in Table I.

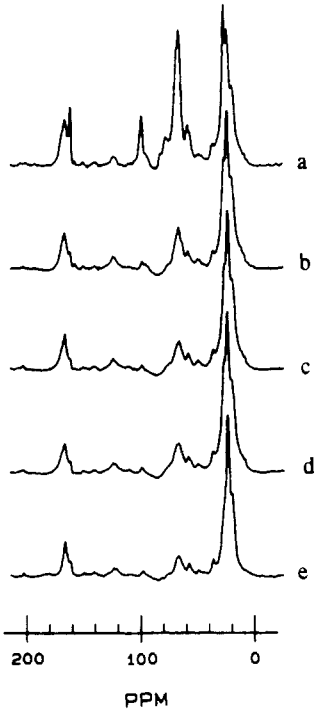

Figure 3. 50.33-MHz ^{13}C CPMAS NMR spectra of samples of lime cuticle after successive purification treatments, obtained with 1.5-ms cross-polarization contact times, 4.8-kHz magic-angle spinning, and high-power ^{1}H decoupling. For the spectra shown, the isolation protocol was complete through the following steps: (a) oxalate treatment; (b) cellulase treatment; (c) pectinase treatment; (d) hemicellulase treatment; (e) Soxhlet extractions. The data were acquired with 6000 transients taken at intervals of 1.0 s, using a spectral width of 20 kHz defined by 2K points. After zero-filling to 4K points, each spectrum was processed with a digital line broadening of 40 Hz and plotted with the largest peak set to full scale.

Hemicellulase Tests: Enzyme and Substrate Concentrations. To optimize the substrate concentration, a series of 45-h reactions was carried out at 37 °C using an enzyme concentration of 49 mg/mL. Locust bean gum (LBG) substrate concentrations were varied between 5 and 125 mg/mL; a plateau in product formation occurred near 120 mg/mL. The enzyme dependence of the hemicellulase reaction was examined in a series of 204-h runs at 37 °C, conducted with an LBG concentration of 120 mg/mL. When the enzyme concentrations were varied between 10 and 185 mg/mL, a broad maximum in galactose production was found at 80 mg/mL enzyme.

Hemicellulase Tests: Time and Temperature. To optimize the reaction time, hydrolyses were conducted at 37 °C, 120 mg/mL substrate, and 78 mg/mL enzyme. For a series of times from 24 to 232 h, a plateau in product formation was found at about 204 h. Finally, the temperature dependence of this reaction was determined from 68-h trials conducted with 120 mg/mL substrate and 78 mg/mL enzyme. Among temperatures from 40 to 50 °C, a maximum yield of hydrolytic products was obtained at 45 °C. The parameters chosen to optimize the hemicellulase reaction appear in Table I.

Plant Polyesters. *Isolation and Spectroscopic Characterization of Lime Fruit Cutin.* To judge the efficacy of successive enzymatic and extractive treatments for the purification of plant polyesters, procedures worked out for the model substrates (Table I) were applied to lime cuticle. The progress of the isolation was monitored both gravimetrically and spectroscopically. Figure 3 shows CPMAS ^{13}C NMR spectra acquired at various stages of the cutin isolation procedure, with the corresponding

removal of carbohydrate and wax impurities amounting to more than 70% of the initial cuticular mass. The NMR spectra show a dramatic and progressive reduction of signal due to cell-wall carbons at 72 and 105 ppm as the enzymatic purification proceeds. The removal of aliphatic waxes, as estimated from the intensity of the peak at 33 ppm (Garbow and Stark, 1990), is evident from examination of the bulk-methylene spectral region. A modest proportion of nitrogen-containing functional groups, from either the cell-wall protein elastin (Fry, 1986) or the biopolymer itself, is evident from elemental analysis (N, 1.5%) but not in the ^{13}C NMR spectra.

Compared with lime cutin that we obtained following previously published protocols (Walton and Kolattukudy, 1972; Deas et al., 1974; Zlotnik-Mazori and Stark, 1988; Garbow and Stark, 1990), the material isolated using the treatments described in Table I has ~25% less spectral intensity at 72 ppm (data not shown). In a prior study of agave leaf samples, resonances at this position were attributed to residual polysaccharides, but evidence for such moieties was absent from pyrolysates analyzed by GC–MS (Nip et al., 1987). Nevertheless, the efficiency of our protocols for breakdown of unsuberized potato tissue (vide infra) makes it reasonable to expect nearly complete removal of the carbohydrates from lime cutin. We therefore assign the remaining NMR signal at 72 ppm to aliphatic carbons of the cutin polyester. The long ^{13}C spin–lattice relaxation time of this carbon signal compares favorably with earlier studies (Garbow and Stark, 1990) and supports a chemical shift assignment of CHOCOR involved in rigid polymer cross-links (Zlotnik-Mazori and Stark, 1988).

Finally, we comment on the narrow carbonyl resonance at 168 ppm, which exhibited anomalous CP behavior and rotating-frame relaxation in the solid state (Zlotnik-Mazori and Stark, 1988). Previously attributed to oxalate or malate (Garbow and Stark, 1990), the assignment of this carbon signal to oxalate has now been confirmed by ^{13}C NMR of an authentic sample (data not shown). A similar conclusion regarding this contaminant can be made from the FTIR spectra of lime cutin shown in Figure 4.

It appears that exogenous oxalate used to separate the cuticle from the epidermal cell wall at the beginning of the isolation procedure becomes trapped within the cutin polymeric matrix. This supposition is reasonable, given the ability of cuticle to absorb ~20% by weight water and organic solvents by swelling (Hartley and Graham-Bryce, 1980; M. Al-Bashir and R. E. Stark, unpublished results). Although successive enzymatic and extractive treatments wash away much of the exogenous oxalate (see Figure 3), traces of this material remain bound to the cutin. A better procedure is to substitute a mild pectinase step (Schonherr et al., 1979), as described above. With this preparative modification, oxalate bands disappear from the IR spectrum (Figure 4), and the two downfield ^{13}C NMR peaks may be attributed with confidence to CH_2OCOR (168 ppm) and CHOCOR (173 ppm) from the cutin polymer.

Isolation and NMR Characterization of Potato Suberin. Compared with cutin, a much greater mass of unsuberized cell walls must be removed to isolate suberized potato periderm. Tables II and III summarize the results for potato tissue grown under different conditions and subjected to different purification protocols. A single cellulase treatment, even using the high enzyme-to-substrate ratio specified in Table II, removes at most 50% of the original dry mass. By contrast, successive cellulase treatments remove 86–96% of this mass.

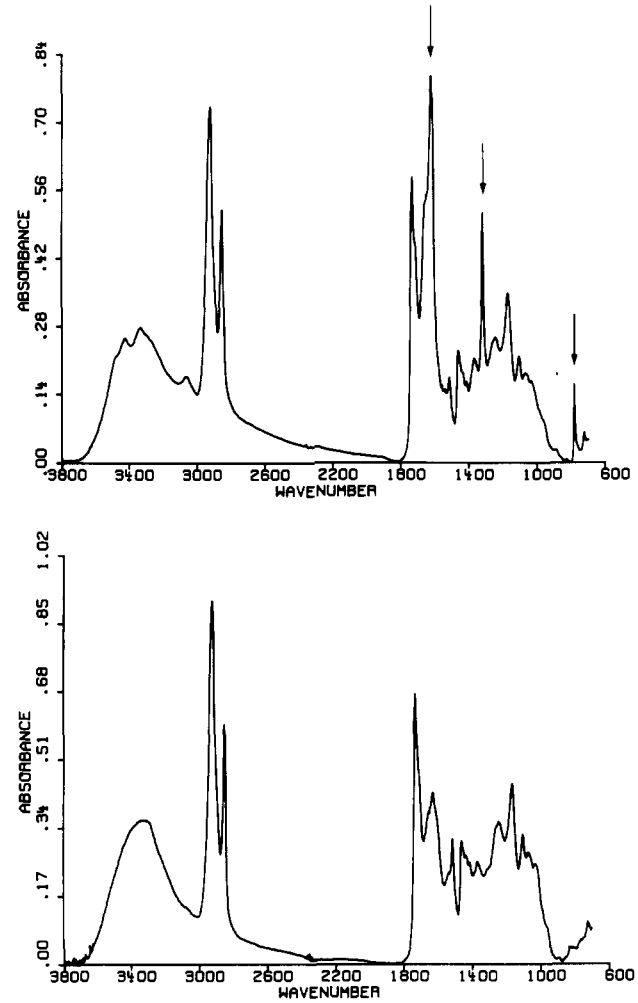

Figure 4. Transmission FTIR spectra of lime cutin isolated using a sodium oxalate procedure (top) and an oxalate-free protocol (bottom). Absorbances from the cutin polyester occur at 3300 cm^{-1} (hydroxyl), 2924 and 2852 cm^{-1} (aliphatic CH stretch), 1731 cm^{-1} (ester carbonyl), 3330 and 1640 cm^{-1} (water), and 1167 cm^{-1} (C–O ester), in agreement with published results for tomato cuticle (Holloway, 1982). The arrows mark three sharp absorbances attributable to oxalate at 1618 cm^{-1} (asymmetric CO_2 stretching vibration), 1316 cm^{-1} (symmetric CO_2 stretching vibration), and 780 cm^{-1} (Ferraro, 1982; Colthup et al., 1975). Additional oxalate bands (3479, 3424, 3333, 3063 cm^{-1}) appear as fine structure on the broad water peak.

The 99% reduction in mass achieved for unsuberized potato provides an estimate for the limiting efficiency of the combined enzymatic and chemical treatment protocol and improves dramatically on estimates of 70% obtained previously using standard enzymatic and extractive methods (Kolattukudy et al., 1975; Garbow et al., 1989). The successful application of cell-wall removal procedures to the potato system, a more difficult extraction than removing cell walls from cutin, supports our previous assumptions about the effectiveness of these procedures in isolations of lime cutin.

By comparison, the five-cellulase protocol alone removes an average of 86% cell wall for potato periderm samples after 1–16 days of wound healing; 93% removal is achieved after subsequent pectinase, hemicellulase, and extractive treatments. The remaining mass may be attributed to suberized tissue that is inaccessible to enzymatic attack. Small sample-to-sample variations in the percentage of solids removed provide a measure of the reproducibility of the growth and isolation procedures.

Figure 5 shows a set of CPMAS ^{13}C NMR spectra collected in parallel with the gravimetric measurements.

Table II. Conditions for Enzymatic Breakdown of Potato Cell Wall[a]

parameter	enzyme				
	cellulase (1)[b]	cellulase (2)[b]	cellulase (n)[b]	pectinase	hemicellulase
substrate concn, mg/mL	34.5	231	31.3	31.3	31.3
enzyme concn, mg/mL	30.8	30.8	2.1	1.3	20.5

[a] Buffer, temperature, and reaction time were as noted in Table I. [b] Numbers in parentheses refer to first treatment, second treatment, and all subsequent treatments, respectively.

Table III. Gravimetric Assessment of Enzymatic and Chemical Treatments for the Isolation of Suberized Potato Cell Walls

last treatment	cell-wall removal,[a] %		
	7-day suberization	no suberization	1–16 day growth[b]
1 cellulase[c]	48	10	42
5 cellulase[c]	91	96	86
7 cellulase[c]	94		
3 Soxhlet		99	93

[a] Percent-by-weight removed from dry potato tissue. Standard treatments (Kolattukudy et al., 1975) removed about 70% (Garbow et al., 1989). Typical isolations started with 50–300 g of wet potato. [b] Average results for 12 time intervals (R. E. Stark, W. Sohn, M. Al-Bashir, R. A. Pacchiano, Jr., and J. R. Garbow, unpublished results). [c] Reaction conditions as in Table II.

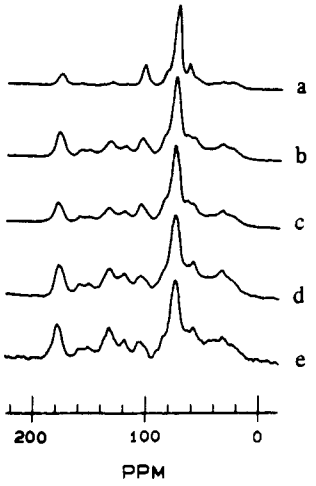

Figure 5. 50.33-MHz ^{13}C CPMAS NMR spectra of 7-day-suberized potato tissue after successive purification treatments, obtained with 1.0-ms cross-polarization contact times, 4.9-kHz magic-angle spinning, and high-power ^{1}H decoupling. For the spectra shown, the isolation protocol was complete through the following steps: (a) one cellulase treatment; (b) five cellulase treatments; (c) pectinase treatment; (d) hemicellulase treatment; (e) Soxhlet extractions. The spectra were acquired with 2400 transients taken at intervals of 1.0 s. The 20-kHz spectral width was defined by 4K frequency-domain points. Each spectrum was processed with a digital line broadening of 80 Hz and plotted with the largest signal set to full scale.

The spectrum acquired for potato tissue after a single cellulase treatment consists primarily of carbohydrate peaks, along with small contributions from the suberin polyester. By contrast, the spectra obtained after subsequent purification steps show a significant enhancement of the suberin ^{13}C NMR signals: carbonyl groups (170 ppm), aromatic moieties (105–150 ppm), and bulk-methylene carbons (20–40 ppm), respectively. As for cutin, minor contributions from nitrogen-containing moieties are indicated by elemental analysis (N, 2.1%). With the present isolation protocol, each major functional group of the biopolyester appears more prominently than in analogous ^{13}C spectra obtained with standard isolation methods (Garbow et al., 1989).

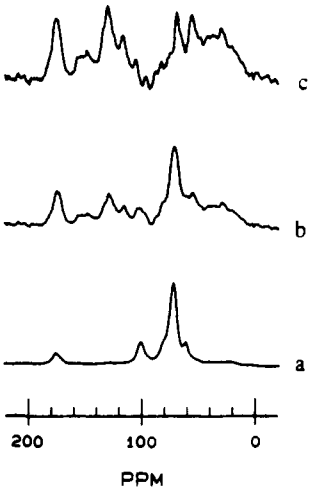

Figure 6. Comparison of ^{13}C NMR spectra for potato tissue after all purification treatments: unsuberized (295 mg, bottom); 7-day-suberized (81 mg, middle); difference (top). Data for both samples were acquired with 1.0-ms cross polarization, 5.0-kHz magic-angle spinning, and high-power decoupling, using 2400 transients and a recycle delay of 1.0 s. The difference spectrum (c) was calculated by subtracting the unsuberized spectrum (a) from the suberized spectrum (b). Negative-going artifacts were minimized. Each spectrum was plotted with its largest signal set to full scale.

Although Table III shows a modest additional loss of mass when more than five cellulase treatments are conducted, the CPMAS ^{13}C NMR spectra are virtually unaffected. Since much of this loss in mass is probably due to filtration and handling operations rather than cellulose hydrolysis, a standard protocol of five treatments was adopted. Each sample was then checked to be sure that cell-wall removal exceeded 85% by weight. If the full complement of purification methods is applied to unsuberized potato tissue, 99% of the sample is hydrolyzed or extracted and the solid-state ^{13}C NMR spectrum exhibits only the expected carbohydrate signals (Figure 6a). The carboxyl resonance near 175 ppm, which has been observed previously in apple fruit cell walls (Irwin, 1989), is attributable primarily to pectin.

Figure 6c shows the difference spectrum generated by subtracting the data from suberized and unsuberized potato cell wall (spectra b and a of Figure 6, respectively). This difference provides a qualitative representation of the ^{13}C NMR spectrum of suberin alone: it has major spectral features corresponding to CH_2O groups at ~65 ppm and OCH_3 groups at 55 ppm, in addition to the phenylpropanoid, aliphatic, and carbonyl resonances identified previously for suberized cell wall. Similar difference spectra have been presented in CPMAS ^{13}C NMR studies of isotopically labeled wheat lignin (Lewis et al., 1987).

CONCLUSION

The ability to generate chemically defined cuticular samples in an efficient and reproducible fashion is clearly

a prerequisite for structural studies of the plant polyesters cutin and suberin. As noted above, high-yield isolation procedures are particularly important when large samples (100–500 mg) are needed for CPMAS ^{13}C NMR spectra or selective depolymerization studies. Moreover, for cuticular materials derived from different chemical treatments or growth conditions, the reliability of information provided by NMR difference spectra will hinge on the consistency of the tissue preparations.

For lime cutin, our results demonstrate that enzymatic protocols optimized to break down model cell-wall substrates can be used to isolate ample quantities of plant material in a straightforward manner. Both CPMAS ^{13}C NMR and FTIR data show that oxalate treatments should be avoided for the removal of pectin, because the oxalate becomes entrapped in the cuticle. CPMAS ^{13}C NMR data show that compared with earlier isolation procedures our series of enzymatic and extractive treatments removes more carbohydrate and wax from the cutin polymer. This conclusion is based upon the diminution of carbohydrate resonances at 72 and 105 ppm and the disappearance of signal due to wax methylene carbons at 33 ppm. It is also supported by gravimetric measurements that indicate successful removal of cell-wall and wax constituents in the potato tissue system. With the availability of such a clean preparation, it becomes possible to focus on cutin's primary and secondary ester linkages or to examine its straight-chain and cross-linked polymeric domains.

For potato suberin, the removal of unsuberized cell wall requires high enzyme-to-substrate ratios and repetitive treatments. The removal of unsuberized cellulose from these tissues is judged complete when at least 85% by weight is lost as soluble degradation products. When this requirement is met and the remainder of the isolation protocol is completed, the CPMAS ^{13}C NMR spectrum of the resulting suberized cell wall has sizeable peaks from carbons of aliphatic–aromatic polyesters, as well as from carbohydrates. The availability of efficient purification protocols for wound-healing plant tissue enables us to use difference spectroscopy to derive the first ^{13}C NMR data for the suberin polyester alone, though the preliminary nature of these results argues for interpretive caution. With an efficient and reproducible source of suberized potato, NMR may be used in structural studies of suberin biosynthesis within the cell wall (R. E. Stark, W. Sohn, R. A. Pacchiano, Jr., M. Al-Bashir, and J. R. Garbow, unpublished results).

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LITERATURE CITED

Colthup, N. B.; Daly, L. H.; Wiberley, S. E. *Introduction to Infrared and Raman Spectroscopy*; Academic Press: New York, 1975; p 304.

Deas, A. H. B.; Baker, E. A.; Holloway, P. J. Identification of 16-hydroxyoxohexadecanoic acid monomers in plant cutins. *Phytochemistry* **1974**, *13*, 1901–1905.

Ferraro, J. R. *The Sadtler Infrared Spectra Handbook of Minerals and Clays*; Sadtler Research Laboratories: Philadelphia, 1982.

Fry, S. C. Cross-linking of matrix polymers in the growing cell walls of angiosperms. *Annu. Rev. Plant Physiol.* **1986**, *37*, 165–186.

Garbow, J. R.; Stark, R. E. Nuclear magnetic resonance relaxation studies of plant polyester dynamics. 1. Cutin from limes. *Macromolecules* **1990**, *23*, 2814–2819.

Garbow, J. R.; Ferrantello, L. M.; Stark, R. E. ^{13}C nuclear magnetic resonance study of suberized potato cell wall. *Plant. Physiol.* **1989**, *90*, 783–787.

Hartley, G. S.; Graham-Bryce, I. J. Penetration of pesticides into higher plants. In *Physical Principles of Pesticide Behavior*; Academic Press: New York, 1980; pp 545–657.

Holloway, P. J. The chemical constitution of plant cutins. In *The Plant Cuticle*; Cutler, D. F., Alvin, K. L., Price, C. E., Eds.; Academic Press: New York, 1982; pp 45–85.

Irwin, P. L. The structure and degradation of intact plant cell wall matrices by ^{13}C CPMAS NMR and related techniques. In *Nuclear Magnetic Resonance in Agriculture*; Pfeffer, P. E., Gerasimowicz, W. V., Eds.; CRC Press: Boca Raton, FL, 1989; pp 337–354.

Kolattukudy, P. E. Biochemistry and function of cutin and suberin. *Can. J. Bot.* **1984**, *62*, 2918–2933.

Kolattukudy, P. E.; Agrawal, V. P. Structure and composition of aliphatic constituents of potato tuber skin (suberin). *Lipids* **1974**, *9*, 682–691.

Kolattukudy, P. E.; Kronman, K.; Poulose, A. J. Determination of structure and composition of suberin from the roots of carrot, parsnip, rutabaga, turnip, red beet, and sweet potato by combined gas-liquid chromatography and mass spectrometry. *Plant. Physiol.* **1975**, *55*, 567–573.

Kolthoff, I. M.; Sandell, E. B. *Textbook of Quantitative Inorganic Analysis*; MacMillan: New York, 1952.

Lewis, N. G.; Yamamoto, E.; Wooten, J. B.; Just, G.; Ohashi, H.; Towers, G. H. N. Monitoring biosynthesis of wheat cell-wall phenylpropanoids in situ. *Science* **1987**, *237*, 1344–1346.

Maciel, G. E.; Haw, J. F.; Smith, D. H.; Gabrielson, B. C.; Hatfield, G. R. Carbon-13 nuclear magnetic resonance of herbaceous plants and their components using cross polarization and magic-angle spinning. *J. Agric. Food Chem.* **1985**, *33*, 185–191.

Nip, M.; de Leeuw, J. W.; Holloway, P. J.; Jensen, J. P. T.; Sprenkels, J. C. M.; de Pooter, M.; Sleeckx, J. J. M. Comparison of flash pyrolysis, differential scanning calorimetry, ^{13}C NMR and IR spectroscopy in the analysis of a highly aliphatic biopolymer from plant cuticles. *J. Anal. Appl. Pyrolysis* **1987**, *11*, 287–295.

Schaefer, J.; Stejskal, E. O. High-resolution ^{13}C NMR of solid polymers. *Top. Carbon-13 NMR Spectrosc.* **1979**, *3*, 283–324.

Schonherr, J.; Eckl, K.; Gruler, H. Water permeability of plant cuticles: the effect of temperature on diffusion of water. *Planta* **1979**, *147*, 21–26.

Stark, R. E.; Garbow, J. R. Nuclear magnetic resonance relaxation studies of plant polyester dynamics. 2. suberized potato cell wall. *Macromolecules* **1992**, *25*, 149–154.

Stark, R. E.; Zlotnik-Mazori, T.; Ferrantello, L. M.; Garbow, J. R. Molecular structure and dynamics of intact plant polyesters: solid-state NMR studies. *ACS Symp. Ser.* **1989**, *No. 399*, 214–229.

Walton, T. J.; Kolattukudy, P. E. Determination of the structures of cutin monomers by a novel depolymerization procedure and combined gas chromatography and mass spectrometry. *Biochemistry* **1972**, *11*, 1885–1896.

Zlotnik-Mazori, T.; Stark, R. E. Nuclear magnetic resonance studies of cutin, an insoluble plant polyester. *Macromolecules* **1988**, *21*, 2412–2417.

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