

Suberin of Potato (*Solanum tuberosum* Var. Nikola): Comparison of the Effect of Cutinase CcCut1 with Chemical Depolymerization

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Chemical and enzymatic depolymerizations of suberin isolated from potato peel (*Solanum tuberosum* var. Nikola) were performed under various conditions. Enzymatic hydrolysis with cutinase CcCut1 and chemical methanolysis with NaOMe of suberin yielded monomeric fragments, which were identified as TMS derivatives with GC-MS and GC-FID. The solid, hydrolysis-resistant residues were analyzed with solid state ¹³C CPMAS NMR, FT-IR, and microscopic methods. Methanolysis released more CHCl₃-soluble material than the cutinase treatment when determined gravimetrically. Interestingly, cutinase-catalyzed hydrolysis produced higher proportions of aliphatic monomers than hydrolysis with the NaOMe procedure when analyzed by GC in the form of TMS derivatives. Monomers released by the two methods were mainly α,ω -dioic acids and ω -hydroxy acids, but the ratios of the detected monomers were different, at 40.0 and 32.7% for methanolysis and 64.6 and 8.2% for cutinase, respectively. Thus, cutinase CcCut1 showed higher activity toward ester bonds of α,ω -dioic acids than toward the bonds of ω -hydroxy acids. The most abundant monomeric compounds were octadec-9-ene-1,18-dioic acid and 18-hydroxyoctadec-9-enoic acid, which accounted for ca. 37 and 28% of all monomers, respectively. The results of the analyses of the chemical and enzymatic hydrolysis products were supported by the spectroscopic analyses with FT-IR and CPMAS ¹³C NMR together with the analysis of the microstructures of the hydrolysis residues by light and confocal microscopy.

KEYWORDS: Cutinase; FT-IR; GC-MS; potato; *Solanum tuberosum*; solid state CPMAS ¹³C NMR; suberin

INTRODUCTION

The periderm of potato (*Solanum tuberosum* L.) tuber forms an effective barrier against desiccation and infection. These functions are achieved mainly by an aliphatic–aromatic cross-linked natural polymer, suberin, which is present in the cell walls of the periderm. Suberized cell walls contain two types of polymeric domains, polyaliphatic and polyphenolic, which are distinct, but covalently linked to each other and to cell wall carbohydrates. Glycerol has been shown to be a linker between these domains, in addition to its role as a linker between monomers in the aliphatic domain. Therefore, glycerol most probably enables the three-dimensional network of the suberin structure. As suberin is attached to the cell wall, it is virtually impossible to isolate it in pure and native state, which further complicates structural studies (1–5).

Most of the knowledge on the structure of the aliphatic domain of suberin is based on the analysis of its depolymerization

products obtained predominantly by ester cleavage (e.g., by alkaline hydrolysis or methanolysis). The quantitative composition of monomeric mixtures obtained depends on the method, but consists mostly of long-chain (16–24 carbon atoms) aliphatic dicarboxylic acids, ω -hydroxy fatty acids, very long chain (20–30 carbons) fatty acids, fatty alcohols, and glycerol together with a small amount of aromatic compounds such as coumaric and ferulic acids (1–5). The distribution of the individual suberin monomeric components and compound classes varies between different plant species and parts of plants. As an example, in some bark suberins, the main compounds, are midchain oxidized acids (bearing epoxy and hydroxy functionalities), which are usually thought to be minor components of other plant suberins. The main differences between suberin and another plant biopolyester, cutin, are the location in the plant and the higher proportions of compounds over 20 carbons in suberin. Also, smaller proportions of epoxy and dihydroxy acids than of diacids have been found to be characteristic for suberin. These properties make suberin and cutin extremely diverse natural sources of various hydroxy fatty acid monomers.

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The composition of the polyphenolic domain of suberin is not as well-known as that of the polyaliphatic domain. This fraction has been studied mainly with spectroscopic methods, for example, FT-IR or solid state NMR (4, 6–10) or degrading methods, such as thioacidolysis (11). These analyses have shown this fraction to contain mostly substituted hydroxycinnamic acids, especially ferulic acid (2, 11–13) with relatively smaller amounts of phenylpropanoid-type structures, similar to lignin monomers (2, 11). Feruloyltyramine, a hydroxycinnamyl amide, has also been detected (14).

Potato suberin has been studied both in native and in wound-induced form. Approximately 22–25% of suberin from the isolated potato peel (treated with cellulase and pectinase and solvent extracted) can be depolymerized by ester cleavage reactions (5, 15, 16). The main suberin monomer groups identified by GC-MS (main monomer in parentheses) are α,ω -dicarboxylic acids 30–50% (octadec-9-ene-1,18-dioic acid) and ω -hydroxy acids 15–30% (18-hydroxyoctadec-9-enoic acid). Some aromatic compounds, fatty alkanols, and fatty acids have also been detected. Glycerol could represent up to ca. 20% of the total monomers released during depolymerization reactions (5), although this has not always been reported in published studies. This is probably due to the various experimental recovery protocols for monomers after depolymerization. Compounds having odd-numbered carbon chains have been identified from suberin monomers, but their amounts are smaller than those of similar even-numbered-chain compounds (16). Hydroxycinnamate-based polymers with some lignin-like components predominate in the polyphenolic domain of natural and wound-induced potato suberin, but differ from each other in composition (8, 9, 11, 13).

In addition to chemical depolymerization reactions, suberin can also be cleaved enzymatically by specific esterases or lipases. Although various cuticular polyesters are abundant constituents of different raw materials, only a limited set of modifying enzymes, other than conventional lipases, are commercially available for the production of such monomers. Cutinases (CUT, EC 3.1.1.74), in particular, can hydrolyze natural cuticular polyesters (cutin, suberin) to lower molecular weight compounds (17, 18). All of the biochemically well-characterized cutinases are esterases, containing the classical SerHisAsp triad common for serine proteases and several lipases, and have a neutral or alkaline pH optimum. Recently, a novel cutinase CcCUT1 of the basidiomycete *Coprinus cinereus* was produced recombinantly, purified, and characterized. CcCUT1 is able to hydrolyze both apple cutin and birch outer bark suberin (17). Due to their broad substrate specificities cutinases have been proposed for various applications for processing diverse raw materials (18). With this perspective, these enzymes are potential tools in the food industry as they may be applied to improve the exploitation of several plant materials and for recovering valuable polyfunctional monomers from polyesters such as suberins and cutins.

Significant amounts of cutin and suberin are present in different food and nonfood byproducts, that is, bark cork, berry, fruit and vegetable processing residues, and cereal byproduct. As an example, around 10% of the fresh weight of potatoes is typically removed during industrial peeling. This industrial waste, composed mainly of skin and tuber flesh, could be a valuable resource of suberin polymers and derived monomers and oligomers. The lack of detailed information on the composition of some of these byproduct materials, and the shortage of suitable enzymes as well as a detailed understanding of the action of those that are available, are the limiting factors for their exploitation. The development of new and more efficient methodologies to isolate suberin/cutin monomeric components, thus ensuring their

wide availability, would certainly provide new opportunities in the search for new applications, such as starting materials for the production of novel macromolecular materials, as recently reviewed by Gandini et al. (3).

As a part of a project aimed at discovering and exploiting lipid-modifying enzymes and their reaction products in industrial food and nonfood processes, the objective of this research was to investigate the effect of the novel cutinase CcCUT1 on the hydrolysis of potato suberin and to compare it with conventional chemical depolymerization methods. The resulting monomers were analyzed chromatographically, and the residues that were resistant to enzymatic and chemical hydrolysis were investigated through further chemical depolymerization and cellulase treatment for better understanding of the structures of the suberized tissues.

MATERIALS AND METHODS

Isolation of Peels and Suberized Tissues. Potatoes (*S. tuberosum* var. Nikola, grown in Karijoki, Finland) harvested in September 2007 and stored at 4 °C for 2 months were peeled using a knife, and the peel was boiled for 1 h and scraped manually with a blade to remove attached flesh. The peel was boiled for another 30 min to remove all of the remaining starch. Isolated potato peel was oven-dried (50–60 °C) and stored in a sealed container.

The suberin-rich membrane fractions were isolated using a method previously described by Kallio et al. (19). Briefly, the procedure was a combination of enzymatic treatments with cellulase (5 g/L Econase CE, AB Enzymes, Darmstadt, Germany) and pectinase (1 g/L Pectinex Ultra SP-L, Novozymes, Bagsvaerd, Denmark) in acetate buffer, followed by exhaustive Soxhlet extraction with CHCl₃ and MeOH (10–12 h each). The procedure was repeated, and the resulting extractive-free suberin-rich membrane, that is, raw suberin (Figure 1), was washed with water and dried.

Chemical Depolymerization. Chemical depolymerization was carried out with methanolysis under three different conditions (Figure 1). Method 1 and 2 reactions were performed in triplicate, and method 3 was performed in duplicate.

Method 1. Dried samples of the raw suberin (20 mg) were treated with 3 mL of freshly prepared 1.0 M NaOMe in dry MeOH. The methanolysis was carried out overnight in sealed tubes in a shaker (approximately 500 rpm) at room temperature. The reaction mixture was acidified with 2 M H₂SO₄ (2.0 mL) in MeOH and the supernatant separated by centrifugation (1315g, 10 min). H₂O (10 mL) was added, and the suberin monomers were extracted with CHCl₃ (2 × 10 mL). The monomer fraction (as methyl esters) in CHCl₃ was dried with anhydrous Na₂SO₄, filtered, and evaporated to dryness. Suberin monomers (monomer 1, Figure 1) were determined gravimetrically and stored in CHCl₃ in a freezer for further analysis.

Method 2. A milder methanolysis reaction was carried out in an overnight reaction of dried samples of raw suberin (20 mg) with 50 mM NaOMe in dry MeOH in a shaker (approximately 500 rpm) at room temperature. The supernatant was separated by centrifugation (1315g 10 min), filtered through a 0.45 μ m filter (Whatman 1, Dassel, Germany), evaporated to dryness, and stored in a desiccator for further analysis (monomer 2, Figure 1).

Method 3. To obtain larger amounts of the depolymerization-resistant solid residue for microscopy, FT-IR, and solid state ¹³C NMR analysis, the residue was isolated by removing suberin by NaOMe-catalyzed methanolysis in reflux. A dried sample of raw suberin (500 mg) was refluxed for 3 h with 100 mL of freshly prepared 1.0 M NaOMe in dry MeOH with magnetic stirring. The reaction mixture was filtered (Whatman, Maidstone, U.K.) and the residue on the filter washed with MeOH until the filtrate was clear and neutral. The residue (residue 1, Figure 1) was further washed with water, oven-dried (50–60 °C), and freeze-dried before analysis. Depolymerized monomers (monomer 3, Figure 1) from this reaction were isolated by CHCl₃ extraction according to the method of Kallio et al. (19).

Enzymatic Hydrolysis. The CcCUT1 produced and characterized previously at VTT Biotechnology (Espoo, Finland) (17) was used in the

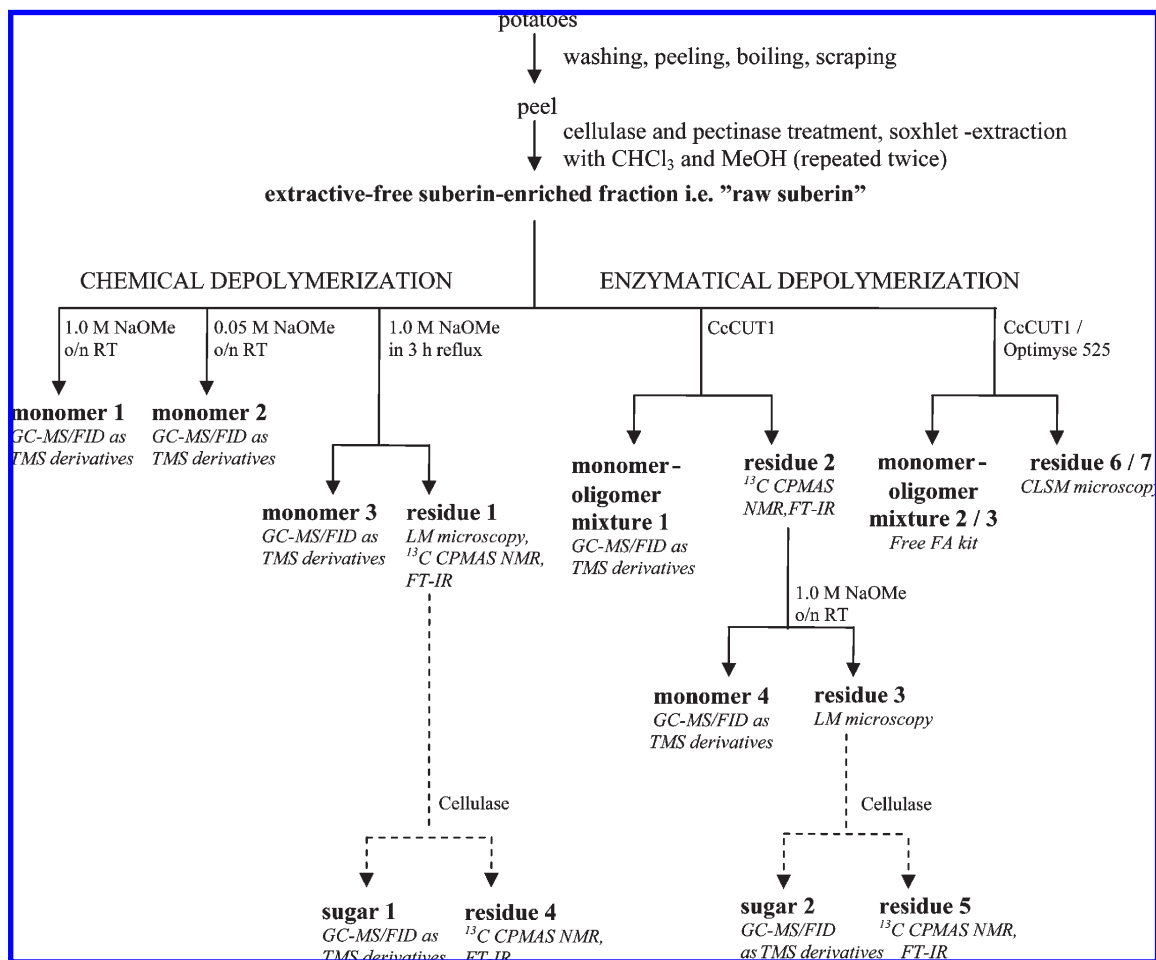


Figure 1. Schematic presentation of the study protocol (**bold** = isolated fraction, *italics* = method of analysis).

enzymatic hydrolysis of suberin. Triplicate samples of raw suberin (50 mg) were finely powdered (Mixer Mill, MM301, Retsch, Haan, Germany) and suspended in 2.5 mL of 0.2 M sodium phosphate buffer, pH 8.0, supplemented with 6.3 mg/L hydrophobin II (20) and 25000 nkat [as *p*-nitrophenyl butyrate esterase activity (17)] of CcCUT1. The suspension was incubated at 50 °C for 48 h under magnetic stirring. CcCUT1 was omitted from the control samples. After incubation, MeOH (0.65 mL) and H₂SO₄ (0.2 mL) were added to the sample, and the product consisting of suberin monomers and oligomers was recovered by three extractions with 2 mL of CHCl₃ each. The chloroform extract was dried with anhydrous Na₂SO₄ and centrifuged, and the CHCl₃-soluble product (monomer-oligomer mix 1, **Figure 1**) was evaporated under a gentle stream of nitrogen. The nonhydrolyzable residue (residue 2, **Figure 1**) was dried. Both products were stored in a desiccator for further analysis.

Products of enzymatic hydrolysis were further analyzed in two separate ways (**Figure 1**).

Method 4. A sample of monomer-oligomer mixture 1 (**Figure 1**) was filtered through a 0.45 μm filter (Whatman, Dassel, Germany), evaporated under a stream of nitrogen and dried in a desiccator.

Method 5. Hydrolysis-resistant residue (residue 2, **Figure 1**) was subjected to 1.0 M NaOMe-catalyzed depolymerization overnight, and monomers were recovered as previously described (monomer 4, **Figure 1**). The residue (residue 3, **Figure 1**) was dried for further analysis.

For microscopic examination, the raw suberin (20 mg mL⁻¹) was incubated in the presence of CcCUT1 as described above, with the exception that the suberin particles were not powdered. As a reference, the suberin was also treated with the Optimyze 525 esterase (Buckman Laboratories, Memphis, TN) under the same conditions as above. After the enzymatic hydrolysis, 50 μL of 6 M HCl was added, and the samples were extracted three times with 2 mL of methyl *tert*-butyl ether (MTBE) to yield monomer-oligomer mixture 2 from CcCUT 1 and monomer-oligomer mixture 3 from Optimyze 525 treatments (**Figure 1**). The

extracts were divided into two 3 mL aliquots for monitoring of the free fatty acids released by hydrolysis. The content of monomeric fatty acids was determined from one aliquot and the total amount of both monomeric and oligomeric fatty acids from the other. For the latter analysis, the oligomers were hydrolyzed by adding 935 μL of 0.5 M KOH in 90% (v/v) ethanol, and the solution was incubated for 1.5 h at 70 °C. The solution was neutralized by adding 60 μL of 6 M HCl. Monomeric fatty acids of both aliquots were determined using the Free Fatty Acids kit (Roche Diagnostics) with palmitic acid as the fatty acid standard and calculated as moles (17). The content of fatty acids in monomeric and oligomeric forms was compared to the total amount of fatty acids released from the suberin after alkaline hydrolysis. After the MTBE extraction of the hydrolysis products, the solid residue (residues 6 and 7, **Figure 1**) was washed with water and subjected to microscopic examination.

Analysis of Suberin Monomers. Suberin monomer fractions from the chemical depolymerization (monomers 1–3, **Figure 1**), cutinase hydrolysis (monomer-oligomer mix 1, **Figure 1**), and CHCl₃-soluble fraction after chemical depolymerization of the cutinase-resistant residue (monomer 4, **Figure 1**) were further trimethylsilylated before chromatographic analysis using the Tri-Sil reagent (HMDS and TMCS in pyridine) (Pierce Chemicals Co., Rockford, IL), after being dried in a desiccator. Following addition of the reagent, the samples were vigorously shaken at room temperature for 5 min and incubated at 60 °C for 15 min. The monomer composition was determined by GC-EI-MS with a Shimadzu GC-MS QP5000 instrument (Shimadzu, Kyoto, Japan) in the following chromatographic conditions: 30 m × 0.25 mm i.d. × 0.25 μm d_f, DB-1 MS column (Agilent J&W, Folsom, CA); split injection (14:1); carrier gas He (linear velocity = 42 cm/s); injector and detector temperatures = 300 °C. The oven temperature was programmed from 100 °C at 10 °C/min to 220 °C, from 220 °C at 3 °C/min to 290 °C, and kept for 15 min at 290 °C. Mass range *m/z* 45–550 was acquired. As a comparison of different detection methods FID (Shimadzu GC 17A, Kyoto, Japan) was used in

Table 1. Gravimetric and Surface Area Results of Potato Peel, Isolated Suberized Membrane, Suberin Monomers, and Depolymerizable-Resistant Residue with Chemical Depolymerization and Cutinase Hydrolysis

		per weight of fresh potato	per area of peel
peel		1.82 mg/g \pm 0.23 mg 1.03 cm ² /g \pm 0.10 cm ²	1.76 mg/cm ² \pm 0.16 mg
isolated suberized membrane (raw suberin)		1.00 mg/g \pm 0.07 mg	0.96 mg/cm ² \pm 0.06 mg
chemical depolymerization	CHCl ₃ -soluble product	0.27 mg/g \pm 0.02 mg	0.26 mg/cm ² \pm 0.02 mg
	residue	0.55 mg/g \pm 0.02 mg	0.53 mg/cm ² \pm 0.02 mg
cutinase hydrolysis	CHCl ₃ -soluble product	0.14 mg/g \pm 0.02 mg	0.14 mg/cm ² \pm 0.02 mg
	residue	0.69 mg/g \pm 0.01 mg	0.66 mg/cm ² \pm 0.01 mg

addition to MS detection, under the conditions described above. All monomer proportions were calculated as average peak area percentage of total peak areas from MS and FID chromatograms.

Compounds were identified by comparing the EI-MS spectra of their TMS derivatives (methyl ester TMS ether or TMS ester TMS ether) with those of reference compounds, with published spectra (21–24), and with typical fragmentation patterns with the aid of retention times. This information has previously been summarized by Kallio et al. (19). The positions of the double bonds were not confirmed by chemical methods. Cholesterol as a TMS derivative was used as an internal standard when determining the relative degree of depolymerization of different fractions.

Sugar Analysis. Residues after chemical depolymerization (residue 1, Figure 1) and cutinase hydrolysis (residue 2, Figure 1) were subjected to a second cellulase treatment in an attempt to remove the remaining, evidently originally shielded, cellulosic material, which was observed in the FT-IR and solid state ¹³C NMR experiments (see Results and Discussion). An aliquot of residue 1 served as a control, without the enzyme in the mixture. Sorbitol was added to each residue as an internal standard, and the sample was incubated overnight at 37 °C with 5 mL of 50 mM acetate buffer (pH 4.0) containing 12 g/L cellulase (Econase CE, AB Enzymes, Darmstadt, Germany). The reaction mixture was separated by centrifugation (1315g, 5 min), and the supernatant was fractionated following a method previously described by Tiitinen et al. (25). A sample of the sugar fraction was evaporated to dryness and dried in a desiccator overnight. TMS derivatives of sugars were prepared by adding Tri-Sil reagent (Pierce, Rockford, IL) into each fraction, followed by vigorous shaking for 5 min and incubation at 60 °C for 30 min. Sugars were analyzed with GC-FID and identified by GC-MS and reference compounds. In addition to sugar fractions 1 and 2 (Figure 1), cellulase-resistant residues (residues 4 and 5, Figure 1) were also obtained and further analyzed after drying.

FT-IR and Solid State ¹³C NMR Analyses. Samples of nondepolymerizable residue from both chemical (residue 1, Figure 1) and enzymatic hydrolysis (residue 2, Figure 1) and the residues remaining after the second cellulase treatment (residues 4 and 5 Figure 1) were subjected to Fourier transform infrared spectroscopy (FT-IR) and solid state ¹³C cross-polarization magic angle spinning (CPMAS) NMR analyses. The FT-IR spectra were recorded with a Spectrum BX FT-IR (Perkin-Elmer, Waltham, MA) equipped with Spectrum v5.3.1 software. FT-IR spectra were measured with a resolution of 4 cm⁻¹ and 16 scans per sample. ¹³C CPMAS spectra were recorded on a Bruker Avance-400 (DRX) NMR spectrometer operating at 400 MHz for proton and at 100 MHz for carbon, using a 7 mm CPMAS Bruker double-bearing probe. Rotors were spun at rates of 5 kHz, and the ¹³C CPMAS spectra were recorded using a proton 90° pulse length of 4.0–4.5 μs, 1 ms contact time, and a recycle time of 5 s. The number of scans depended on the amount of sample available and is stated in the caption of Figure 4 separately for each sample.

Microscopic Analyses. Enzymatic modifications of raw suberin with CcCUT 1 and Optimize 525 were visualized using confocal laser scanning microscopy (CLSM, residues 6 and 7, Figure 1) equipment consisting of a Bio-Rad Radiance Plus confocal scanning system (Bio-Rad, Hemel Hempstead, Hertfordshire, U.K.) attached to a Nikon Eclipse E600 microscope (Nikon Corp., Tokyo, Japan). For imaging, samples were stained in 0.01% (w/v) Nile Blue (Gurr Products, Romford, Essex, U.K.) for 2 min and rinsed with water. Surfaces and newly cut cross sections of the material were examined using a 488 nm argon laser line for excitation

and band-pass filters at 500–560 and 575–625 nm for green and red fluorescence, respectively. 3D green- and red-filtered emission images of the optical sections were obtained using either a 10× objective (Nikon Plan Apo, numerical aperture 0.45) to the depth of 40 μm with a 4.0 μm z step, 1.0 μm pixel size, and resolution of 512 × 512 or a 20× objective (Nikon Plan Apo, numerical aperture 0.75) to the depth of 24 μm with 2.0 μm z step, 0.5 μm pixel size, and resolution of 512 × 512. The final CLSM micrographs were reconstructed by the superimposition of a green-filtered emission image and a red-filtered emission image, in which suberin-containing cell walls appeared yellow.

Residue 1 from chemical depolymerization and residue 3 from cutinase hydrolysis followed by chemical depolymerization were analyzed by light microscopy (LM, Figure 1) due to their fine particle size, which made them unsuitable for confocal microscopy. The samples for LM analysis were prepared as previously described by Olkku et al. (26). Briefly, the samples were fixed in 1% glutaraldehyde in 0.1 M phosphate buffer (pH 7), dehydrated with ethanol, embedded in Historesin, and sectioned (2 μm) with a rotary microtome. For microscopic examination the sections were stained with 0.5% Oil Red O (BDH Chemicals, Poole, Dorset, U.K.) in 70% ethanol for 1 min. Suberin-containing structures appear as yellow-orange stained structures in the micrographs.

An overview of the study protocol and all of the obtained products is shown in Figure 1.

RESULTS AND DISCUSSION

Isolation of Suberized Membrane Fraction (Raw Suberin), Chemical Depolymerization, and Cutinase Hydrolysis. The surface area of the potato tubers varied according to their size and was between 79 and 140 cm² per tuber. The average results of gravimetric and surface area measurements of potato peel, raw suberin, chemical depolymerization and cutinase hydrolysis products (monomer 1 and monomer–oligomer mix 1, Figure 1), and the residues from both reactions (residues 1 and 2, Figure 1) are shown in Table 1.

The mass of washed potato peel was on average 0.18% of the tuber fresh weight. The raw suberin accounted for 55% (SD 4%) of peel and ca. 0.1% of potato fresh weight. Suberin monomer 1, released by 1.0 M NaOMe-catalyzed methanolysis, corresponded to 27% (SD 2%) of the total suberin, leaving 56% (SD 2%) of the nondepolymerizable residue, the remaining 18% (SD 4%) being water-soluble material. The gravimetric results from the overnight reaction were comparable to results from the 3 h reflux reaction (monomer 3, 27%, SD 3%; residue 1, 57%, SD 1%; water-soluble, 16%, SD 1%), which resulted in complete depolymerization of the ester-bound suberin according to FT-IR and solid state ¹³C NMR (see below). These results are consistent with previously published information for potato suberin (5, 15).

Enzymatic hydrolysis with cutinase CcCUT 1 yielded 15% (SD 2%) of CHCl₃-soluble product (monomer–oligomer mix 1, Figure 1). The residue from cutinase hydrolysis (residue 2), which accounted for 72% (SD 1%) of the total, was subjected to chemical depolymerization, yielding an additional 11% of CHCl₃-soluble monomers and a residue (residue 3). These

combined yields together are comparable with the yield obtained by chemical depolymerization.

Suberin Monomeric Composition. The composition of suberin monomers, released from the raw suberin by methanolysis or by CcCUT 1 hydrolysis, was identified on the basis of GC retention times and EI-mass spectra as TMS ethers of methyl or TMS esters. Only a few reference compounds were available (given in **Table 2**), and thus the retention behaviors and the mass spectral fragmentation patterns were the main methods of identification (19, 21–24). The diagnostic ions and retention times of each derivative are shown in **Table 2**.

To find out, in general, how effectively raw suberin was depolymerized with the various methods (**Figure 1**), the yields of the released soluble fractions were determined gravimetrically. The derivatized monomers were further analyzed by GC using cholesterol as an internal standard. The chromatographic results were calculated by comparing the total peak area of all analytes to the peak area of cholesterol (**Figure 2**). Even though correction factors for the monomer derivatives were not determined due to the lack of reference compounds, relative differences between the five fractions could be defined. The results also revealed that all of the methods left, in addition to the polymeric fraction, soluble remnant compounds that were not detected in GC analysis. This was in accordance with earlier published results (4, 5).

The most effective depolymerization took place with 1.0 M NaOMe-catalyzed methanolysis, yielding almost twice the amount of CHCl_3 -soluble compounds produced in the cutinase hydrolysis. Interestingly, the results from the GC analysis showed that the enzymatic hydrolysis produced relatively more of the compounds analyzable with GC even though the total degree of depolymerization was clearly lower. Thus, cutinase treatment seems to be a more selective method for releasing aliphatic hydroxy fatty acids and dicarboxylic acids from suberin than the chemical methods, indicating the potential for specific industrial purposes.

Results from the analysis of suberin monomers (**Table 3**) obtained from the peel of potato variety Nikola by 1.0 M NaOMe-catalyzed methanolysis were similar to results found in the literature for other potato varieties (e.g., Irmgard, Russet Burbank) in which different depolymerization techniques were used (8, 21, 22). The most abundant compound groups were α,ω -diacids and ω -hydroxy acids, accounting together for >70% of monomers, with small proportions of fatty acids, alcohols, and aromatic compounds. The most abundant compounds were octadec-9-ene-1,18-dioic acid and 18-hydroxyoctadec-9-enoic acid, which accounted ca. for 37 and 28%, respectively, of all monomers. These monomers have been reported to be the most abundant compounds in potato suberin (5, 15, 16). All monomer proportions were calculated as the average peak area percent of total peak areas from MS (**Figure 3**) and FID chromatograms. Some short-chain (4–9 carbon chain) α,ω -diacids and ω -hydroxy acids, which have also been reported for cork suberin (4), were also detected in the studied samples.

Methanolysis catalyzed by 1.0 M NaOMe at room temperature overnight yielded a similar relative proportion of monomers as the 3 h reaction in reflux. With lower NaOMe concentration the composition of the hydrolysis mixture was substantially different; for example, the amount of ω -hydroxy acids was lower and epoxy acids were absent. Similar results have previously been reported for suberin depolymerization with several different NaOMe concentrations (4, 27). Glycerol has been reported to be an important unit of suberin (2, 3, 5), but depending on the method used it might not be quantifiable as it may be discarded due to its water solubility. With the milder NaOMe concentration, the water–chloroform partitioning after methanolysis could be

omitted, and the amount of glycerol determined (ca. 20% of total peak areas in GC-MS and FID chromatograms) was consistent with earlier results from potato suberin hydrolysis (variety not known) (5). With the exception of glycerol, the most abundant compounds were the same when different concentrations of NaOMe were used for methanolysis (**Table 3**).

The monomers released by CcCut1 (monomer–oligomer mix 1, **Figure 1**) were the same as the monomers from chemical depolymerization, but the ratio of ω -hydroxy acids to α,ω -diacids was significantly lower in enzymatic hydrolysis (**Table 3**). Epoxy-substituted monomers were absent in cutinase hydrolysis products, just as they were in the milder NaOMe methanolysis. The residue from the enzymatic hydrolysis (residue 2, **Figure 1**) was also subjected to chemical depolymerization to solubilize the remaining ester-bound suberin monomers, which were shown to exist in the product by FT-IR and solid state ^{13}C NMR (see later in the text). This reaction yielded monomer fraction 4 (**Figure 1**), which contained more ω -hydroxy acids compared to α,ω -diacids and more unidentified compounds than the other methods (**Table 3**). About 7% of hydrocarbons were also detected by GC-MS, but were absent in all other experiments.

The results of the monomer analysis of the cutinase-containing reaction mixtures showed a clear predominance of α,ω -diacids over ω -hydroxy acids when compared with chemical depolymerization with 1.0 M NaOMe (**Table 3**). Furthermore, the results obtained from the cutinase hydrolysis were more similar to those obtained with milder methanolysis, but relatively more of the cutinase product can be analyzed with GC in the form of TMS derivatives (**Figure 2**). These results together indicate that cutinase has a higher specificity for the hydrolysis of α,ω -diacids rather than ω -hydroxy acids. This could be because the former are mainly involved in more labile glyceryl–ester bonds, whereas the latter are in the form of less reactive wax-type ester bonds (1–4). In addition, the prevalence of the α,ω -diacids over ω -hydroxy acids could also be the result of the various organizations of the units in the polymer and the various amounts of cross-linkages. Also, the absence of released epoxy acids under milder conditions of chemical depolymerization or by cutinase hydrolysis may be explained by their hindered structure and location in the suberin layers. It is also possible that this may be due to opening of the epoxy rings due to the method of hydrolysis. In the case of cutinase-catalyzed hydrolysis, this produces a small rise in the corresponding dihydroxydic acids and trihydroxy fatty acids, although this does not totally explain the differences.

Solid State ^{13}C NMR and FT-IR Analysis of Depolymerization-Resistant Residues. Additional information on the mode of action of the cutinase CcCUT1 compared with chemical depolymerization can be achieved from the solid residues obtained from the reactions. Due to their insoluble nature, the residues from chemical depolymerization (residue 1, **Figure 1**) and cutinase hydrolysis (residue 2, **Figure 1**) were analyzed by solid state ^{13}C CPMAS NMR and by FT-IR. Both techniques have previously been used successfully, for example, in cork and potato suberin analysis (4, 6–10), one study tackling, in particular, the effects of suberin removal (10). In these studies, comparative spectra from unreacted, that is, raw suberin with depolymerization residue and suberin extracts have been published and differences reviewed.

The ^{13}C CPMAS NMR and FT-IR spectra of these residues (**Figures 4** and **5**) confirm the results obtained from the other analyses and from previous observations on similar residues (9, 10). The spectra of residue 1 (**Figure 4A**) showed a clear predominant cellulose profile (64 ppm C_6 ; 72–74 ppm $\text{C}_2, \text{C}_3, \text{C}_5$; 82–90 ppm C_4 ; 105 ppm C_1) overlapping with resonances from lignin-like structures (56 ppm $-\text{OCH}_3$; 62 ppm $-\text{CH}_2\text{O}-$;

Table 2. Diagnostic Ions of CHCl_3 -Soluble Long-Chain Suberin Monomers from Potato

compound/ derivative	retention time and mass spectral cleavage of each derivative						
	t_R	Di Me ester, TMS ether m/z	t_R	Me ester TMS ester, TMS ether m/z	t_R	Di TMS ester, TMS ether m/z	ID
α,ω -dioic acids							
hexadecane-1,16-dioic acid	16.9	283 [M - 31] ⁺ , 241, 209, 191, 166, 154, 135, 125, 112, 98, 84, 74, 69, 55	19.2	357 [M - 15] ⁺ , 307, 269, 159, 146, 129, 117, 98, 75, 73, 69, 55	21.6	430 [M] ⁺ , 415 [M - 15] ⁺ , 299, 217, 204, 170, 147, 129, 117, 75, 73, 55	MS
octadec-9-ene-1,18-dioic acid	20.0	340 [M] ⁺ , 309 [M - 31] ⁺ , 308, 290, 276, 98, 95, 81, 67, 55	22.4	383 [M - 15] ⁺ , 333, 295, 159, 129, 117, 98, 95, 81, 75, 73, 67, 55	24.9	456 [M] ⁺ , 441 [M - 15] ⁺ , 217, 204, 170, 147, 129, 117, 75, 73, 55	MS
octadecane-1,18-dioic acid	20.6	311 [M - 31] ⁺ , 269, 237, 196, 154, 140, 126, 112, 98, 84, 74, 69, 55	23.0	385 [M - 15] ⁺ , 335, 297, 159, 146, 129, 117, 98, 83, 75, 73, 67, 55	25.6	458 [M] ⁺ , 443 [M - 15] ⁺ , 327, 311, 217, 204, 170, 147, 129, 117, 75, 73, 55	MS
9,10-dihydroxyoctadecane-1,18-dioic acid	27.1	332, 259, 243, 155, 147, 129, 109, 73, 55	29.5	390, 317, 259, 217, 204, 155, 147, 129, 109, 73, 55	31.8	390, 317, 301, 227, 217, 204, 147, 129, 109, 73, 55	MS
9,10-epoxyoctadecane-1,18-dioic acid ^a	26.2	274, 259, 243, 227, 155, 137, 129, 109, 89, 73, 55	28.6	332, 317, 259, 217, 204, 155, 137, 129, 109, 73, 55	31.0	341, 329, 317, 309, 217, 204, 147, 129, 109, 75, 73, 55	MS
eicosane-1,20-dioic acid	24.5	339 [M - 31] ⁺ , 297, 265, 154, 125, 112, 98, 84, 74, 55	27.2	413 [M - 15] ⁺ , 363, 325, 159, 146, 129, 117, 98, 83, 75, 73, 67, 55	29.9	486 [M] ⁺ , 471 [M - 15] ⁺ , 355, 217, 204, 147, 129, 117, 75, 73, 55	MS
docosane-1,22-dioic acid	28.8	367 [M - 31] ⁺ , 325, 293, 154, 135, 125, 112, 98, 84, 74, 55	31.5	441 [M - 15] ⁺ , 391, 353, 159, 146, 129, 117, 98, 83, 75, 73, 67, 55	34.0	514 [M] ⁺ , 499 [M - 15] ⁺ , 383, 217, 204, 147, 129, 117, 75, 73, 55	MS
tetracosane-1,24-dioic acid	33.2	395 [M - 31] ⁺ , 353, 321, 154, 135, 125, 112, 98, 84, 74, 55	35.8	469 [M - 15] ⁺ , 419, 381, 159, 146, 129, 117, 98, 83, 75, 73, 67, 55	38.5	542 [M] ⁺ , 527 [M - 15] ⁺ , 411, 217, 204, 170, 147, 129, 117, 75, 73, 55	MS
hexacosane-1,26-dioic acid	37.7	423 [M - 31] ⁺ , 381, 349, 154, 135, 125, 112, 98, 84, 74, 55	40.8	497 [M - 15] ⁺ , 447, 409, 159, 146, 129, 117, 98, 83, 75, 73, 67, 55	44.5	570 [M] ⁺ , 555 [M - 15] ⁺ , 439, 217, 204, 147, 129, 117, 75, 73, 55	MS
octacosane-1,28-dioic acid	43.6	451 [M - 31] ⁺ , 409, 377, 154, 135, 112, 98, 84, 74, 55	47.9	525 [M - 15] ⁺ , 475, 437, 159, 146, 129, 117, 98, 75, 73, 55		598 [M] ⁺ , 583 [M - 15] ⁺ , 467, 217, 204, 147, 129, 117, 75, 73, 55	MS
ω -hydroxy acids							
16-hydroxyhexadecanoic acid							
	17.8	343 [M - 15] ⁺ , 327 [M - 31] ⁺ , 311 [M - 47] ⁺ , 159, 146, 103, 89, 75, 73, 55	20.0	401 [M - 15] ⁺ , 385 [M - 31] ⁺ , 311 [M - 105] ⁺ , 217, 204, 147, 117, 103, 75, 73, 55			R, MS
18-hydroxyoctadecadienoic acid	20.4	367 [M - 15] ⁺ , 335 [M - 47] ⁺ , 149, 135, 129, 107, 95, 75, 73, 67, 55		440 [M] ⁺ , 425 [M - 15] ⁺ , 409 [M - 31] ⁺ , 335 [M - 105] ⁺ , 217, 204, 147, 135, 129, 121, 117, 109, 95, 75, 73, 55			MS
18-hydroxyoctadec-9-enoic acid	20.9	384 [M] ⁺ , 369 [M - 15] ⁺ , 353 [M - 31] ⁺ , 337 [M - 47] ⁺ , 159, 146, 129, 109, 95, 75, 73, 55	23.2	442 [M] ⁺ , 427 [M - 15] ⁺ , 411 [M - 31] ⁺ , 337 [M - 105] ⁺ , 383, 217, 204, 147, 135, 129, 117, 109, 95, 75, 73, 55			MS
18-hydroxyoctadecanoic acid	21.4	339 [M - 47] ⁺ , 159, 146, 129, 107, 95, 75, 73, 55		444 [M] ⁺ , 429 [M - 15] ⁺ , 413 [M - 31] ⁺ , 339 [M - 105] ⁺ , 217, 204, 147, 129, 117, 103, 75, 73, 55			MS
9,10-epoxy-18-hydroxyoctadecanoic acid ^a	26.9	489 [M - 15] ⁺ , 473 [M - 31] ⁺ , 457 [M - 47] ⁺ , 385, 303, 274, 259, 201, 155, 129, 121, 109, 103, 81, 75, 73, 55	29.4	547 [M - 15] ⁺ , 443, 425, 332, 317, 303, 243, 147, 129, 121, 109, 103, 75, 73, 67			R, MS
9,10,18-trihydroxyoctadecanoic acid	27.8	547 [M - 15] ⁺ , 332, 303, 259, 243, 212, 155, 147, 129, 109, 103, 81, 75, 73	30.3	515 [M - 105] ⁺ , 390, 317, 303, 217, 204, 147, 129, 109, 103, 81, 75, 73			MS
20-hydroxyeicosanoic acid	25.5	399 [M - 15] ⁺ , 383 [M - 31] ⁺ , 367 [M - 47] ⁺ , 159, 146, 103, 89, 75, 73, 55		457 [M - 15] ⁺ , 441 [M - 31] ⁺ , 367 [M - 105] ⁺ , 217, 204, 147, 129, 117, 103, 75, 73, 55			MS
22-hydroxydocosanoic acid	29.7	442 [M] ⁺ , 427 [M - 15] ⁺ , 411 [M - 31] ⁺ , 395 [M - 47] ⁺ , 159, 146, 129, 103, 83, 75, 73, 55	32.3	485 [M - 15] ⁺ , 469 [M - 31] ⁺ , 395 [M - 105] ⁺ , 217, 204, 147, 129, 117, 103, 75, 73, 55			R, MS
24-hydroxytetracosanoic acid	34.0	470 [M] ⁺ , 455 [M - 15] ⁺ , 439 [M - 31] ⁺ , 423 [M - 47] ⁺ , 159, 146, 129, 103, 87, 75, 73, 55	36.5	513 [M - 15] ⁺ , 497 [M - 31] ⁺ , 423 [M - 105] ⁺ , 217, 204, 147, 129, 117, 103, 75, 73, 55			MS
26-hydroxyhexacosanoic acid	38.5	483 [M - 15] ⁺ , 467 [M - 31] ⁺ , 451 [M - 47] ⁺ , 159, 146, 129, 103, 87, 75, 73, 55	41.7	541 [M - 15] ⁺ , 525 [M - 31] ⁺ , 451 [M - 105] ⁺ , 217, 204, 147, 129, 117, 103, 75, 73, 55			MS
28-hydroxyoctacosanoic acid	44.6	511 [M - 15] ⁺ , 495 [M - 31] ⁺ , 479 [M - 47] ⁺ , 159, 146, 129, 103, 87, 75, 73, 55	49.1	569 [M - 15] ⁺ , 553 [M - 31] ⁺ , 479 [M - 105] ⁺ , 217, 204, 147, 129, 117, 103, 75, 73, 55			MS

Table 2. Continued

aromatics	t_R	Me ester, TMS ether m/z	t_R	TMS ester, TMS ether m/z	ID
4-hydroxy-3-methoxybenzaldehyde (vanillin)	7.8	224 [M] ⁺ , 209 [M - 15] ⁺ , 194, 179, 165, 163, 137, 104, 89, 73, 59			MS
4-hydroxy-3-methoxybenzoic acid (vanillic acid)	9.3	254 [M] ⁺ , 239 [M - 15] ⁺ , 224, 193, 179, 165, 137, 104, 89, 73, 59	10.8	312 [M] ⁺ , 297 [M - 15] ⁺ , 282, 267, 253, 223, 193, 179, 165, 147, 137, 126, 89, 73, 59	MS
coumaric acid	10.9	250 [M] ⁺ , 235 [M - 15] ⁺ , 219 [M - 31] ⁺ , 203, 192, 175, 161, 135, 115, 102, 89, 73, 59	12.9	308 [M] ⁺ , 293 [M - 15] ⁺ , 249, 219, 179, 147, 73, 59	R, MS
ferulic acid (2 peaks)	11.4 + 12.8	280 [M] ⁺ , 265 [M - 15] ⁺ , 250, 219, 191, 175, 160, 117, 102, 89, 73, 59	12.6 + 14.8	338 [M] ⁺ , 323 [M - 15] ⁺ , 308, 293, 279, 249, 219, 191, 175, 160, 147, 117, 102, 89, 73, 59	R, MS

^a Identified as corresponding methoxyhydrin compounds. ID, method of identification: R, reference compound; MS, mass spectral cleavage pattern together with retention behavior and published spectra. Positions of double bond were not confirmed by chemical methods.

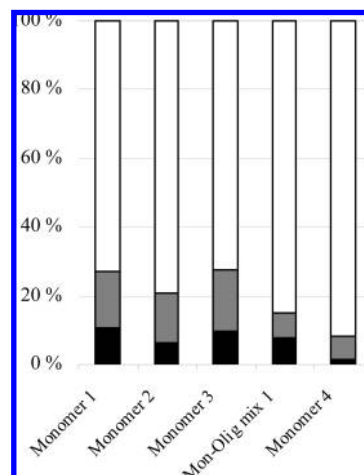


Figure 2. Hydrolysis yield of the chloroform-soluble compounds detected by gravimetric analysis (gray + black) and proportion of the monomeric compounds detected by GC analysis (black) released from the raw suberin samples (100%).

83 ppm CHOAr ; 90 ppm CHO- , 105–150 ppm arom -CH- and quaternary C) (6, 7, 9, 10). The signals at 170–180 ppm should arise from carbonyl resonances possibly present in aromatic structures and/or polysaccharides such as pectin or hemicellulose or, eventually, of suberin components. The corresponding FT-IR spectrum (Figure 5A) confirmed the absence of ester-bonded aliphatic structures (no band at 1750 cm^{-1} ester C=O), due to chemical depolymerization (residue 1, Figure 1). The FT-IR fingerprint region was consistent with previously published polysaccharide spectra and confirmed the predominant polysaccharide nature of residue 1 ($1000\text{--}1250\text{ cm}^{-1}$ CO stretching of polysaccharides). Lignin-like aromatic structures (1607 , 1513 cm^{-1} aromatic C=C), some aliphatic chains (2930 cm^{-1} CH), and aliphatic olefinic groups (1630 cm^{-1} C=C) were also found to be present in smaller proportions (7).

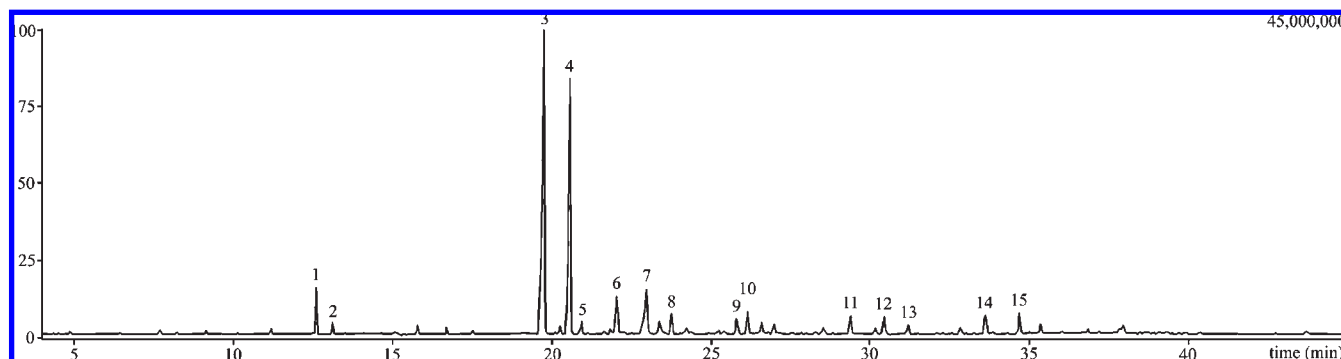
With regard to residue 2 (Figure 4B), the polysaccharide NMR profile is significantly less intense compared to the clearer signals at around 20–40 ppm, assignable to methylene carbons of suberin (6, 7, 9, 10) and the carbonyl resonance at 170–180 ppm. In addition, a small signal is also observed at 130 ppm, which could be due to unsaturated carbons in the suberin domain, which is compatible with the predominance of long-chain unsaturated fatty acids in the monomeric composition of the suberin samples studied. This cutinase hydrolysis residue (Figure 5B) showed a FT-IR profile different from that of residue 1, the main difference being the presence of the strong peaks at 1138 and 1112 cm^{-1} found not to derive from cellulose, but most probably from lignin type structures (CH, CO deformation), in agreement with the relative increase in lignin type structures as viewed by NMR. Furthermore, the FT-IR spectra of this cutinase hydrolysis residue also confirmed the presence of aliphatic CH moieties of suberin-derived structures, in accordance with the other results. The presence of carbonyl groups based on the NMR results could only be confirmed in the FT-IR spectra by the presence of a shoulder at $1740\text{--}1720\text{ cm}^{-1}$.

Sugar Monomer and Residue Analysis after Cellulase Treatment. Solid residues from cutinase hydrolysis (residue 2, Figure 1) and chemical depolymerization (residue 1, Figure 1) were subjected to cellulase treatment, because the FT-IR and solid state ^{13}C NMR analyses suggested that polysaccharides were the predominant components of the residues. Given that the potato peel was previously treated with cellulase, the presence of polysaccharides could be due either to their incomplete removal during the

Table 3. CHCl₃-Soluble Long-Chain Suberin Monomers from Potato Variety Nikola Released by Different Depolymerization Techniques (Percent Values Are Average Percent of Total Peak Area from GC-MS and GC-FID Chromatograms)^a

compound	chemical depolymerization						cutinase hydrolysis			
	monomer 1		monomer 2		monomer 3		monomer—oligomer mix 1		monomer 4	
	%	SD	%	SD	%	SD	%	SD	%	SD
α,ω-dioic acids	39.98	0.39	51.03	4.00	38.20	2.90	63.29	3.41	12.76	0.80
hexadecane-1,16-dioic acid	0.36	0.04	0.56	0.19	0.40	0.03	0.70	0.13	0.11	0.03
octadec-9-ene-1,18-dioic acid (3)	36.62	0.32	47.41	4.02	33.73	1.98	59.67	3.62	12.07	0.68
octadecane-1,18-dioic acid	0.51	0.02	0.55	0.08	0.43	0.08	0.72	0.07	0.19	0.02
9,10-dihydroxyoctadecane-1,18-dioic acid	0.13	0.04	—	—	0.13	0.07	0.54	0.07	0.25	0.04
9,10-epoxyoctadecane-1,18-dioic acid (9) ^b	1.18	0.03	—	—	1.30	0.51	—	—	0.19	—
eicosane-1,20-dioic acid	ns	—	0.45	0.10	ns	—	—	—	—	—
docosane-1,22-dioic acid	0.49	0.03	0.32	0.05	0.36	0.04	0.36	0.08	0.20	—
tetracosane-1,24-dioic acid	0.49	0.02	0.44	0.29	0.37	0.11	0.40	0.10	—	—
hexacosane-1,26-dioic acid	0.20	0.02	—	—	0.80	0.76	0.14	0.05	—	—
octacosane-1,28-dioic acid	tr	—	—	—	0.19	0.08	0.12	—	—	—
ω-hydroxy acids	32.71	0.36	24.25	3.38	32.76	1.68	7.96	1.20	38.82	2.46
16-hydroxyhexadecanoic acid	0.25	0.02	0.32	0.09	0.30	0.06	0.11	0.06	0.56	0.03
18-hydroxyoctadecadienoic acid	0.17	0.03	—	—	0.11	0.03	—	—	0.20	0.05
18-hydroxyoctadec-9-enoic acid (4)	27.61	0.32	21.63	3.43	26.84	1.84	6.57	1.31	33.30	2.75
18-hydroxyoctadecanoic acid	tr	—	—	—	tr	—	—	—	0.11	0.02
9,10-epoxy-18-hydroxyoctadecanoic acid ^b	0.77	0.03	—	—	0.89	0.07	—	—	0.11	—
9,10,18-trihydroxyoctadecanoic acid	0.12	0.01	0.10	0.02	0.15	0.03	0.20	0.02	0.28	0.13
20-hydroxyeicosanoic acid	tr	—	—	—	tr	—	—	—	0.07	—
22-hydroxydocosanoic acid (11)	1.31	0.05	0.70	0.20	1.28	0.05	0.53	0.17	1.57	0.25
24-hydroxytetracosanoic acid (14)	1.51	0.02	—	—	1.51	0.09	0.45	0.03	2.00	0.48
26-hydroxyhexacosanoic acid	0.66	0.02	0.71	0.08	0.95	0.01	0.20	0.04	0.92	0.22
28-hydroxyoctacosanoic acid	0.25	0.03	—	—	0.47	0.09	—	—	0.38	0.07
aromatics	2.27	0.27	2.43	0.52	2.58	0.21	2.05	0.50	1.49	0.65
4-hydroxy-3-methoxybenzaldehyde	tr	—	0.18	0.02	0.26	0.03	ns	—	ns	—
4-hydroxy-3-methoxybenzoic acid	0.10	0.05	0.20	0.08	0.14	0.01	0.08	0.04	—	—
coumaric acid	tr	—	0.13	0.03	tr	—	—	—	tr	—
ferulic acid (1)	2.03	0.24	1.99	0.39	2.14	0.15	1.98	0.47	1.47	0.64
fatty alkanols and diols (2, 5, 13)	4.51	0.31	3.30	0.60	8.10	1.07	0.69	0.14	10.26	0.17
fatty acids (10, 12, 15)	6.29	0.23	3.87	0.31	9.42	1.46	9.41	0.99	7.95	0.49
hydrocarbons	—	—	—	—	—	—	—	—	6.73	2.34
unidentified (6, 7, 8)	14.28	0.20	15.11	1.26	8.97	1.85	16.60	3.01	22.00	1.35
total	100	—	100	—	100	—	100	—	100	—

^a SD, standard deviation; —, not detected; ns, not separated from unidentified peak; tr, <0.1%. Number in parentheses after the compound name refers to peak identifications in **Figure 3**. ^b Identified as corresponding methoxyhydrin compounds. Positions of double bond not confirmed by chemical methods.

**Figure 3.** Total ion chromatogram from GC-MS of monomer 1 (**Figure 1**). For peak identification see retention times in **Table 2** and numbers for major peaks in **Table 3**.

isolation procedure or to the shielding effect of suberin polyaliphatic layers, which hindered cellulase hydrolysis. Residue 1 was also treated as a control, which confirmed that there were no side reactions in the process without the cellulase (measured with FT-IR and solid state ¹³C NMR). GC sugar analysis of the reaction products confirmed glucose as the main product (>90%), followed by minor amounts of xylose and arabinose

(together accounting for 5–10% of the area of glucose peaks). Compounds were identified with the aid of the mass spectra of reference compounds and retention behavior. Noncellulosic residues from this reaction represented about 50 and 70% of the original mass of the cutinase hydrolysis residue and the chemical depolymerization residue, respectively. These residues were analyzed with solid state ¹³C CP MAS NMR techniques and

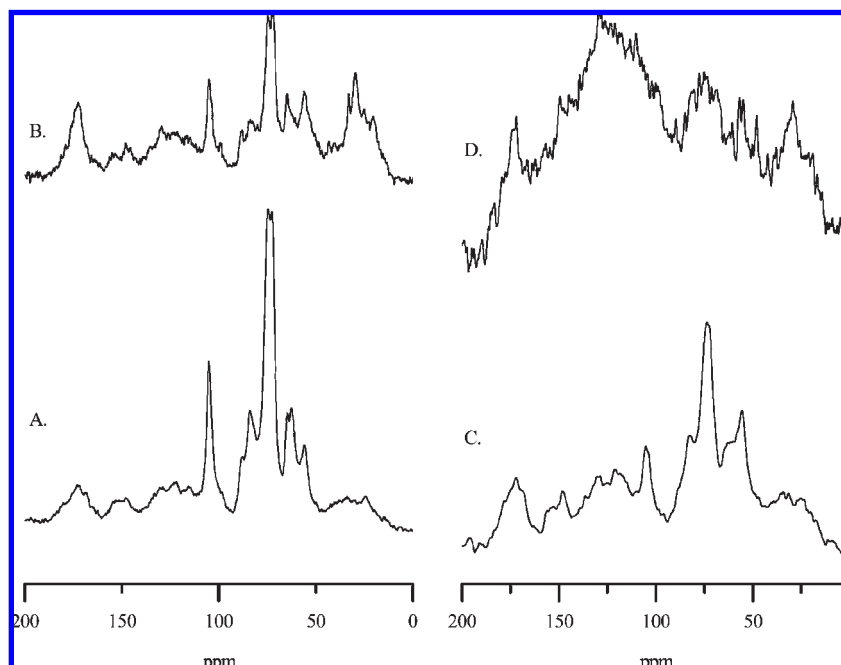


Figure 4. ^{13}C CP MAS NMR spectra of (A) chemical depolymerization residue (residue 1, **Figure 1**), ns = 6300; (B) cutinase hydrolysis residue (residue 2, **Figure 1**), ns = 28650; (C) cellulase-treated chemical depolymerization residue (residue 4, **Figure 1**), ns = 1980; and (D) cellulase-treated cutinase hydrolysis residue (residue 5, **Figure 1**), ns = 16540.

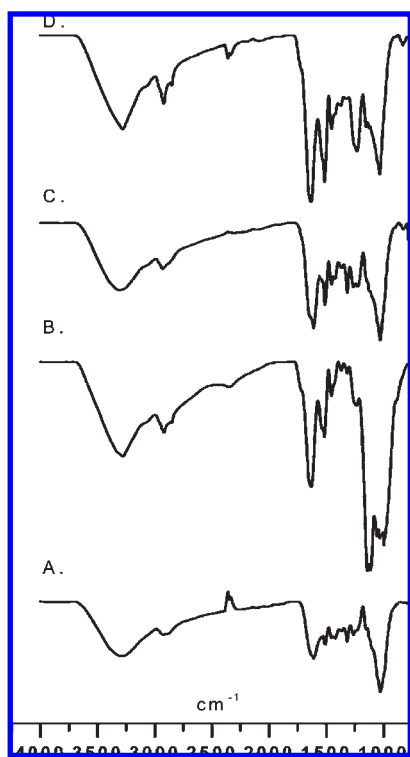


Figure 5. FT-IR spectra of (A) chemical depolymerization residue (residue 1, **Figure 1**); (B) cutinase hydrolysis residue (residue 2, **Figure 1**); (C) cellulase-treated chemical depolymerization residue (residue 4, **Figure 1**); (D) cellulase-treated cutinase hydrolysis residue (residue 5, **Figure 1**).

by FT-IR, and the spectra obtained were compared with those of the residues before cellulase treatment.

The ^{13}C NMR spectra of the cellulase-treated residues (**Figure 4C,D**) clearly had less intense resonances from polysaccharides, as expected. Interestingly, the cellulase-treated residue

from the cutinase treatment (residue 5, **Figure 4D**) shows that complete cellulose removal was achieved, the spectrum showing only broad aromatic resonances (100–155 ppm), with residual resonances in other spectral regions. This broad spectrum is consistent with the total collapse of cellular structure upon the additional cellulase treatment, leading to a more amorphous and disorganized structure. The corresponding FT-IR spectrum (**Figure 5D**) also changes significantly, with the 1138 and 1112 cm^{-1} peaks being removed, leaving peaks at 1630, 1513, and 1230 cm^{-1} predominating, which suggests that lignin-like structure was significantly affected. On the other hand, the FT-IR spectrum of residue 4 (**Figure 5C**) still confirms the presence of all components, although in different proportions.

Microscopy of the Residues. Raw suberin fractions were treated both with cutinase CcCUT1 and with Optimyze 525, examined with confocal microscopy (CLMS, **Figure 6**), and compared with the raw suberin fraction without any enzymatic treatment. In this study, suberized cell walls were localized using lipophilic Nile Blue stain. The active ingredient in Nile Blue is its fluorescent oxidation product called Nile Red, which is chemically a neutral benzophenoxazine. Nile Red is a general lipid stain, but it shows the most intense fluorescence for nonpolar solvents such as neutral lipids (28–30). Suberin detection using Nile Red is probably based on its attachment to the aliphatic part of the suberin macromolecules.

According to the confocal microscopic analysis, both enzymes CcCUT1 and Optimyze effectively hydrolyzed the suberized cell walls in the raw suberin samples (**Figure 6**). In all samples, suberin appeared as yellow lamellar structures and the cellular network of potato periderm was apparent. In the reference samples, suberin was seen as a continuous lamellar component surrounding the cork cells (**Figure 6A,D**). After enzyme treatment with the cutinase CcCUT1 (residue 6, **Figure 1**), suberin layers were only weakly visible when imaged from the top of the sample. In the cross section, suberin lamellae still showed the cellular network in the middle of the sample, but the outer layers of the sample had reduced fluorescence for suberin when compared to the reference

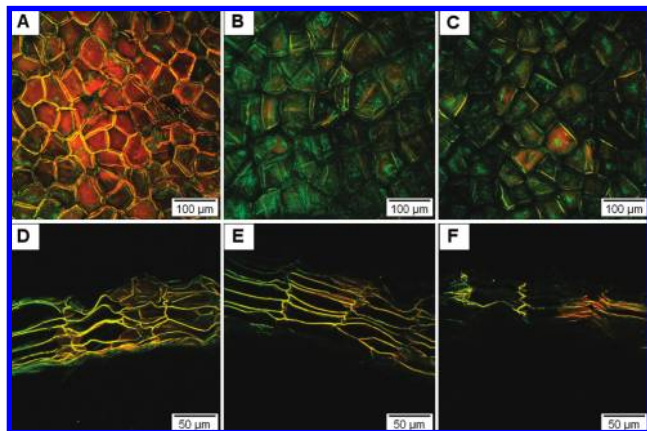


Figure 6. Microstructure of isolated potato suberin (A–C from top, D–F cross section) after cutinase treatments with CcCUT1 (B, E) and Optimyze (C, F). Reference sample (A, D) was incubated without enzyme (50 °C, pH 8, 24 h, 500 nkat L⁻¹, 6.3 mg L⁻¹ hydrophobin II, MTBE extraction).

(Figure 6B,E). Fairly similar effects were obtained in the sample treated with the Optimyze 525 enzyme preparation (residue 7, Figure 1). However, suberin seemed to be more efficiently hydrolyzed with Optimyze 525, as some cell walls did not show any fluorescence at all but some were still brightly fluorescent (Figure 6C,F). The composition of the red-stained layer observed on the surface of the suberized potato membrane, which was also affected by the enzymes, is not known (Figure 6A). It may contain esterified waxes or other fatty substances as it was clearly affected by the enzyme treatments.

In CLSM micrographs, suberin was expected to be localized in the structures that were stained yellow, because they were the sites that showed fluorescence through all of the emission spectra expected for the lipid stain used. Structures seen in the micrographs as green or red might contain fatty substances, as it is known that the emission maximum of Nile Red stain depends on the lipid composition (30). However, their emission spectra differed from those of the yellow structures recognized as suberin lamellae, even though they were affected by the enzyme treatments. On the basis of the microscopic data, it seems that chemical depolymerization results in more effective depolymerization of suberin than the enzymatic procedures with CcCut1 and Optimyze. These data support the information obtained from chromatographic and spectroscopic analysis.

Residues from chemical depolymerization (residue 1, Figure 1) and those from cutinase hydrolysis that were further depolymerized by methanolysis (residue 3, Figure 1) were analyzed by light microscopy, because their fine particle size was not suitable for confocal microscopy. The microscopic examination revealed that residues 1 and 3 (Figure 7) did not contain any structures showing bright orange fluorescence typical of the suberin-containing particles stained with Oil Red O (Figure 7). However, the lamellar, slightly brownish structures observed in the samples may be the remains of the suberized cell walls left after the depolymerization treatments.

Monitoring of Cutinase Hydrolysis by the Release of Monomeric and Oligomeric Fatty Acids. The enzymatic hydrolysis by CcCUT1 and Optimyze 525 of the samples subjected to microscopic analysis (residue 6 and 7, Figure 1) was also analyzed by determining the fatty acids released (monomer–oligomer mix 2, Figure 1). The Free Fatty Acids Kit (Roche) used is limited to fatty acids with carbon chain lengths between C₈ and C₁₈ and does not therefore necessarily represent the total amount of suberin monomers released. The method can nevertheless be used

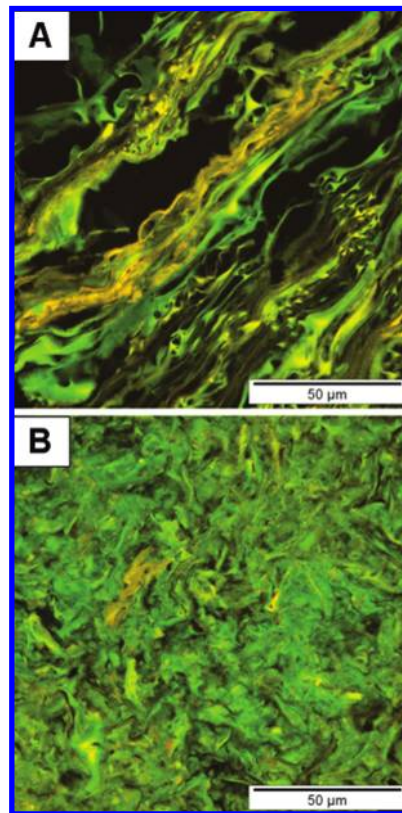


Figure 7. Microstructure of modified potato suberin fractions stained with Oil Red O and imaged using epifluorescence microscopy: (A) potato suberin residue after chemical depolymerization (residue 1, Figure 1); (B) residue after cutinase hydrolysis and chemical depolymerization (residue 3, Figure 1).

for estimating the amounts of oligomers and monomers released. As shown in Table 4, monomeric fatty acids were released from the raw suberin with equal efficiency by Optimyze 525 and CcCUT1. However, as calculated from the values presented in Table 4, more suberin oligomers were released by Optimyze 525 treatment (approximately 31% mol/mol) than by treatment with CcCUT1 (approximately 11% mol/mol). This result corresponds with the results from the microscopic analysis described above.

The hydrolytic action of CcCUT1 has previously been investigated with birch outer bark suberin and apple cutin as the substrates (17). Roughly 2 times more fatty acids (as moles) was released from suberin than from cutin. The amount of fatty acids released per mass of substrate (0.22 μmol mg⁻¹ of both monomeric and oligomeric fatty acids) in the presence of CcCUT1 in the present study was of the same order of magnitude as the amount of released fatty acids reported previously (0.29 μmol mg⁻¹ of both monomeric and oligomeric), although less enzyme (one-fifth) and a shorter incubation time (half) were used in the previous study. It is thus possible that suberin is in general more recalcitrant toward hydrolysis by CcCUT1 than the true substrate, cutin.

The combined chemical and enzymatic procedures made it possible to compare the two unit operations with each other, even though none of the hydrolytic steps were quantitative. GC analysis of the monomers of the suberin hydrolysates, sugar analysis of the evident cellulose polymer, and FT-IR and solid state ¹³CNMR analyses supplemented with the microscopic investigations gave new information of the inexactly defined raw suberin fraction of potato peels. Cutinase treatment seemed to leave some of the ester bonds unattacked, and methanolysis

Table 4. Monomeric and Oligomeric Fatty Acids Released from Isolated Suberin during Enzymatic Hydrolysis^a

enzyme	monomeric fatty acids ^b		monomeric and oligomeric fatty acids ^{b,c}	
	$\mu\text{mol}/\text{mg}$	% ($\mu\text{mol}/\mu\text{mol}$)	$\mu\text{mol}/\text{mg}$	% ($\mu\text{mol}/\mu\text{mol}$)
reference	0.00	0	0.00	0
CcCUT1	0.18	46	0.22	57
Optimzye	0.16	42	0.28	73

^aThe values presented refer to those fatty acids that can be detected with the Fatty Acids Kit. Errors were <10%. ^bPer mass of isolated suberin. ^cAfter alkali hydrolysis of the oligomers.

released significantly more monomeric fragments than the enzymatic treatment. The proportion of dioic acids was clearly dominating in the enzymatic hydrolysate.

We have not tested specificities and activities of different cutinases or esterases on the raw suberin fraction of potato peels. The analytical scheme including chemical hydrolysis and cutinase and cellulose treatments gives us, however, an indication that the formation of the potato peel during storage might occur via formation of alternating layers. This could, in turn, be verified by “peeling” the raw suberin fraction at different time periods during one year with repeated chemical, esterase, and cellulase treatment steps. The few microscopic investigations of ours supported the relevance of this strategy.

ABBREVIATIONS USED

NaOMe, sodium methoxide; TMS, trimethylsilyl; GC-MS, gas chromatography–mass spectrometry; FID, flame ionization detector; NMR, nuclear magnetic resonance; FT-IR, Fourier transform infrared; MTBE, methyl *tert*-butyl ether; HMDS, hexamethyldisilazane; TMCS, trimethylchlorosilane; CPMAS, cross-polarization magic angle spinning.

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