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Structure of the Polyphenolic Component of Suberin Isolated from Potato (*Solanum tuberosum* var. Nikola)

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Suberin is present in the underground parts of vegetables and in the bark of trees. Characterization of suberin and the structure of its polyphenolic component have been hampered by insolubility of the polymers. Thus, enzymatically isolated and extractive free suberin enriched fraction from potato, *Solanum tuberosum* var. Nikola, and the chemically further fractionated phenolics were characterized in solid state by FTIR, DSC, and elemental analysis to identify the groups and to verify success of isolation. For MW and quantitative determination of the groups, polymers were solubilized in ionic liquid derivatized and analyzed by GPC and ^{31}P NMR. Suberin enriched fraction, MW = ca. 44 $\times$ 10^3 g/mol, is a mixture of carbohydrates and polyesters of aliphatic long chain hydroxy fatty acids and diacids linked via ester bonds to the phenolics, MW = ca. 27 $\times$ 10^3 g/mol, formed by guaiacyl- and *p*-hydroxyphenyl structures. Phenolics in peels may be important sources of antioxidants for various applications.

KEYWORDS: ionic liquid; phenolic component; ^{31}P NMR; potato; structure suberin

INTRODUCTION

Plants require a hydrophobic water-tight layer on the cell wall surface to protect them from desiccation as well as from gases and fluxes of water solutions containing air pollutants and other harmful substances. The outer layer plays an important role in protecting plants from biotic and abiotic attack and also controls plant morphology. Water resistance is typically imparted by a wax layer, but the wax needs a polymeric network to support the structure of the layer. These polymers present in cell wall barriers are called cutin and suberin (*1*−*4*). Cutin is formed in plant primary tissues, such as in the epidermis cells of leaves and fruits, and suberin is formed in secondary tissues, such as in the periderm phellem cells as barks in trees, and underground tubers as in the case of potatoes. Suberin is considered to be a part of the cell wall, as a true secondary wall, because in most of the cases it is deposited inside the primary layer of the cell wall. By contrast, cutin is considered to be an extracellular material because it is deposited outside of the first formed primary wall (*5*). Suberin has also been found in other parts of plants where exceptionally high aqueous impermeability is required, such as forks and knot holes. Although these polymers are ubiquitous in plants, the biosynthesis, structure, and function of suberin and cutin are the least understood of the major plant polymers (*6*).

During plant growth, suberin is deposited constitutively in both external and internal tissues at specific cell wall locations, and hence it is an integral part of the cell wall structure (*4*, *7*). However, suberin deposits have also been found in the anticlinal primary cell walls as well as during an early stage of endodermis development (*8*). This explains why suberin is difficult, perhaps even impossible, to isolate pure and in its native state, which has hampered structural characterization of the insoluble polymer (*9*). Suberin isolation has been performed mainly using methods developed originally for cutin and lignin separation. After physical treatments, such as peeling, skinning, and boiling, the suberized cells are isolated from the plant surface by hydrolytic enzymes, such as cellulases and pectinases, which depolymerize most of the carbohydrate-containing materials. Next, exhaustive solvent extraction treatment is typically used to remove soluble waxes and fatty acids associated with suberin (*4*, *10*). However, in most cases, polymeric carbohydrates cannot be completely removed by these treatments. Thus, for example, in the case of cork suberin, attempts have been made to improve the yield and purity of the polymer by combined enzymatic and solvent treatments, although with limited success (*11*). Hence, during recent years, most of the structural characterizations of suberin have focused on the wound-induced suberization of potato periderm (*12*).

At present, it is assumed that in plant cell walls suberin exists as a thick lamellae-like structure. Several structural models aiming to describe the macromolecular structure of suberin as well as the assembly of different polymeric components in suberized membranes have been presented during recent years (*1*, *13*−*15*). According to Bernards (*1*), suberin is composed of an aliphatic polyester fraction and a fraction of C−C and ether-linked aromatics that are highly resistant to enzymatic and chemical depolymerization. These two covalently linked components of suberin are usually referred to as "polyaliphatic" and "polyaromatic" domains, respectively (*1*−*3*). Although the monomer

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composition of suberin is rather well-documented for various plants and tissues, the structure of the polyphenolic polymer is not yet fully understood. Recently, the analysis of plant lines with modified cutins and suberins has revealed interaction between these two different types of polymers. However, the major questions concerning the isolation, structure, and function of suberin still remain unresolved (*16*).

The structures of suberin enriched fraction and its phenolic component isolated enzymatically and chemically from potato (*Solanum tuberosum* var. Nikola) were studied in solution and in solid form in order to elucidate the potential of the uncharacterized polymer for various applications. The suberin studied in this work was formed in the potatoes during normal storage of the tubers, unlike the widely examined wound-induced suberin typically formed on the surfaces of chopped potatoes during storage of the tubers. Recently, a new method based on dissolution of lignocellulosic material in ionic liquids, such as 1-allyl-3-methylimidazolium chloride ([Amim]Cl), followed by phosphitylation reaction (*17*) and ^{31}P NMR measurement (*18*) has proved to be an extremely powerful technique for the quantitative determination of various hydroxyl groups, such as alcohols, phenolics, and carboxylic acids, present in these highly insoluble biopolymers. In addition, gel permeation chromatography (GPC) was used to determine the molecular weights of the polymers benzoylated in ionic liquids (our unpublished method). FTIR, differential scanning calorimetry (DSC), and element analysis were used to characterize the structures of the polymers in the solid state.

MATERIALS AND METHODS

Isolation of Suberin and Its Phenolic Component from Potato. Potatoes (*Solanum tuberosum*) of the variety Nikola grown in 2007 in Karijoki (Finland) were mechanically peeled, and the separated peels were boiled for 1 h and scraped manually to remove the residual attached flesh. The peels were then further boiled for 30 min to remove all the remaining starch. Finally, the isolated peels were dried at 50−60 °C and stored in a desiccator.

Before structural characterization, the purified peels were further fractionated analogously to the isolation of berry cutin and potato suberin, as described recently by Kallio et al. (*19*) and Järvinen et al. (*20*). The dried peels were treated with 6 g/L of cellulase (AB Enzymes, Darmstadt, Germany) and 1.2 g/L of pectinases (Novozymes, Bagswaerd, Denmark) in acetate buffer. Exhaustive extraction in a Soxhlet apparatus was first performed using chloroform followed by methanol, 10−12 h each. Enzymatic treatment and solvent extraction were repeated to yield pure extractive free suberized potato membranes, which were washed with Milli-Q water and dried before the final depolymerization step. In the following, enzymatically fractionated extractive free potato membranes are called raw suberin.

The phenolic component of suberin was isolated by chemical depolymerization. Methanolysis of raw suberin was performed by refluxing 500 mg of dried material for 3 h with 100 mL of freshly prepared 1.0 M NaOMe in dry methanol with magnetic stirring. The reaction mixture was passed through filter paper #1 (Whatman GmbH, Dassel, Germany), and the residue on the filter was washed with methanol until the filtrate was clear and the pH of the solution was neutral. Finally, the residue was washed with Milli-Q water and dried at 50−60 °C.

Benzoylation of Raw Suberin and the Phenolic Component. The method described below was developed by the group of Argyropoulos following the principles established in the work described in the reference (*21*). Ionic liquid (i.e., 950 mg of [Amim]Cl) was mixed separately with raw suberin, and the pulverized phenolic component (ca. 40 mg, accurately weighed) in 15 mL round-bottom glass bottles was vortexed until all visible solid particles had dispersed. The samples were heated at 80 °C and mixed with a magnetic stirrer overnight until clear, transparent solutions were obtained. Pyridine (150 μL) was added to each reaction mixture, and the solutions were vigorously vortexed until visibly homogeneous solutions were obtained. The samples were cooled to room temperature, and 200 μL of benzoyl chloride (Sigma-Aldrich, St. Louis, MO) was added in one portion to each sample. The resulting reaction mixtures were vortexed until formation of homogeneous white pastes was observed. The samples were heated at 40 °C for 3 h with continuous mixing. Finally, a mixture of 2.5 mL of deionized water and 7.5 mL of EtOH was added to the reaction mixtures, which were shaken vigorously for 5 min. The precipitates were filtered off with sintered funnels (medium grade), washed with 30 mL of EtOH, scraped off from the filter, and purified with 15 mL of MeOH under constant stirring overnight without heating. The resulting brown solid materials were filtered off from the methanol solution, dried, and characterized by FTIR spectroscopy.

FTIR Spectroscopy. FTIR spectra of isolated and benzoylated raw suberin as well as of the phenolic component were measured using Equinox 55 FTIR spectrometer equipped with an IR microscope and mercury cadmium telluride detector (Bruker, Karlsruhe, Germany). A few milligrams of accurately weighed dried polymers were applied to a diamond cell, and the transmission spectra were measured from 4000 to 600 cm^{-1} at room temperature. The spectral resolution was maintained at 4 cm^{-1}, and the number of scans was typically 100. Opus software was used for data processing as well as for visualization of the measured FTIR spectra. The spectra were normalized according to the highest peak in the spectrum.

Differential Scanning Calorimetry. Phase transitions of raw suberin and the phenolic component were determined by a DSC 820 (Mettler, Dietikon, Switzerland) differential scanning calorimeter equipped with a liquid nitrogen cooling system. Samples of ca. 5 mg were accurately weighed in aluminum pans and sealed. The temperature program was started at 0 °C and increased to 130 °C at a heating rate of 10 °C/min. Endothermal enthalpy changes were integrated and calculated from the DSC curves as J/g of the dried sample.

Elemental Analysis. The percentage contents of carbon (C), hydrogen (H), and nitrogen (N) in raw suberin and the phenolic component were determined from the dried samples using an element analyzer (Perkin-Elmer, Norwalk, CT). The remaining parts of the samples were assumed to be oxygen. The protein contents of raw suberin and the phenolic component were estimated from the nitrogen contents of the biopolymers by multiplying the obtained experimental values by 6.25 (*21*).

Quantitative ^{31}P NMR Spectroscopy. The following method is essentially identical to that of King et al (*22*). Before ^{31}P NMR analysis, ca. 0.6 g of raw suberin and 0.2 g of phenolic component were pulverized in an Ultrasonic homogenizer (Torrey Hills Technologies, San Diego, CA) in zirconia jars (diameter = 54 mm, depth = 46 mm) using three large and four small zirconia balls. The time for the ball-milling was 2 × 2 h, with a 30 min pause between the millings to avoid overheating. The number of revolutions per min was 400 rpm. Then ball-milled samples of raw suberin and the phenolic component (ca. 25 mg of each accurately weighed) were magnetically stirred with 0.6 g of [Amim]Cl ionic liquid at 80 °C for 18 h to obtain clear, transparent, and homogeneous solutions. For phosphorylation, 150 μL of pyridine was added in one portion to the reaction mixtures, and the samples were vortexed until visibly homogeneous. The reaction mixtures were cooled to room temperature, and an excess of freshly synthesized 2-chloro-4,4,5,5-tetramethyl-1,3,2-dioxaphospholane reagent (PR[II]) was added in one portion to the mixtures. The samples were vortexed until visibly homogeneous cream pastes were formed. Then 500 μL of chromium(III)acetylacetonate (Cr[acac]$_3$), solubilized in deuterated chloroform ($CDCl_3$) at a concentration of 1 mg/mL, was added in four 125 μL portions to the reaction mixtures, which were vortexed between the additions. Finally, 125 μL of internal standard [i.e., *endo-N*-hydroxy-5-norbornene-2,3-dicarboximide solution (121.5 mM dissolved in a 3:2 ratio of pyridine/$CDCl_3$)] was added in one portion, and the reaction mixtures were vigorously vortexed. The phosphorylated raw suberin and the phenolic component solutions were further diluted with Cr[acac]$_3$ solvent, first to 1 mL and then to 2 and 3 mL volumes.

^{31}P NMR spectra of the phosphorylated raw suberin and phenolic component were recorded from 700 μL sample volumes using a 5 mm o.d. NMR tube. The quantitative spectra were acquired immediately after sample preparation using a Bruker 300 MHz spectrometer (Bruker, Newark, DE) equipped with a quadruple probe dedicated to detection of ^{31}P, ^{13}C, ^{19}F, and ^{1}H nuclei. Acquisition of NMR spectra of modified raw suberin and phenolic component was performed using published procedures (*17*, *18*). A total of 1024 scans were acquired for each sample with a relaxation delay time of 5.0 s to ensure sufficient resolution and to get a good signal-to-noise ratio. All spectra were calibrated with a known

signal derived from the adduct of phosphitylated water at 132.2 ppm. Complete phosphitylation of the polymers was confirmed for each dilution by monitoring the broad peak arising from the unreacted phosphitylation reagent located at 175.9 ppm in the spectrum.

Gel Permeation Chromatography. For molecular weight determination of raw suberin and the phenolic component, 5 mg of benzoylated raw suberin and phenolic component was solubilized in 2 mL of 100% THF (Riedel-de-Haën, Seelze, Germany) and injected into Styragel HR 5E and HR 1 columns (Waters Corporation, Milford, MA) linked in series. The chromatographic system was calibrated with polystyrene standards with molecular weights from 0.82 to 1860 kg/mol (Sigma-Aldrich, St. Louis, MO). Separations of the modified polymers were performed on a Waters chromatographic system (Waters Corporation, Milford, MA) equipped with a UV detector operating at 280 nm. The isocratic chromatographic separations were carried out at 30 °C using 100% THF as eluent. Flow rate was 0.7 mL/min. Millennium 32 GPC software (Waters Corporation, Milford, MA) was used for data acquisition and processing.

RESULTS AND DISCUSSION

Isolation of Polymers. It is assumed that the aliphatic and aromatic components of suberin are heterogeneously organized in suberized plant cells in lamellar-like structures, as shown by Bernards (*1*). Thus, isolation of the suberin and phenolic component in the native state is an extremely demanding task. By using the enzymatic and chemical fractionation methods, the yield of the isolated extractive free suberized membrane (i.e., raw suberin) was 55% of the dried peel weight and ca. 0.1% of the fresh weight of the potato. The amount of the non-depolymerizable phenolic component present in raw suberin was 57%.

Characterization of Raw Suberin and the Phenolic Component in the Solid State. The isolated components were first characterized in the solid state. The FTIR spectrum of raw suberin (**Figure 1A**) revealed that it contains ester functionalities and aliphatic hydrocarbon chains derived from hydroxy fatty acid−diacid polyester, carbohydrates, and phenolics as previously detected in our recent studies (*20*). To facilitate comparison between raw suberin and the phenolic component, the corresponding FTIR spectra were overlaid in **Figure 1A**. In the spectrum of raw suberin, bands of the ester bonds of the hydroxy fatty acid and diacid polymers could be detected at 1742 (C═O), 1243, and 1261 cm^{-1} (−C−O−C−). These bands were missing in the spectrum measured from the phenolic component. Moreover, intense −C−H vibrations of $-CH_3$ and $-CH_2$ groups at 1460, 1318, and 780 cm^{-1} of the long aliphatic hydrocarbon chain could be identified in the raw suberin spectrum, but less clearly in the spectrum measured from the phenolic component. In addition, in the suberin spectrum, strong vibrations of C−H bonds of $-CH_3$ and $-CH_2$ groups could be detected from 2800 to 3000 cm^{-1} (data not shown). However, these bands were substantially decreased in the spectrum measured from phenolic component, also indicating success of the fractionation. In the case of both polymers, a very strong broad band from 980 to 1200 cm^{-1} was identified, which is indicative of −C−O−C− vibration of sugar rings of the cellulosics. In addition, the so-called "lignin triplet", that is, three bands from 1400 to 1600 cm^{-1} derived from C═C vibration of the aromatic ring, could be identified in the spectrum of raw suberin and the phenolic component. The two broad bands at 1651 and 1621 cm^{-1} most probably resulted from the water associated with isolated polymers and/or from the conjugated carbonyl groups.

The elemental analysis of raw suberin and phenolic component showed only very small differences between the polymers. The carbon, hydrogen, and nitrogen contents of raw suberin were 50.5, 2.24, and 2.42%, respectively. The rest was assumed to be oxygen (i.e., 44.4%). The protein content was estimated from the amount of nitrogen (protein content = $N \times 6.25$), and it was found to be ca. 15% for suberin. For the phenolic component, the

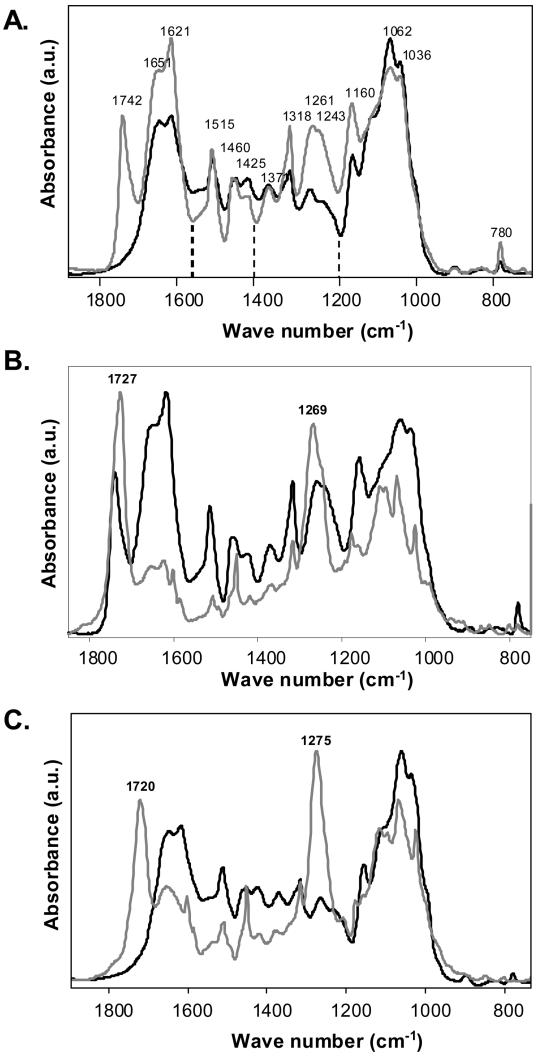

Figure 1. FTIR spectra measured from raw suberin and the phenolic component. (**A**) Spectrum measured from the phenolic component (black) overlaid with the spectrum measured from raw suberin (gray). (**B**) Spectrum measured from the native raw suberin (black) overlaid with the spectrum measured from benzoylated suberin (gray). (**C**) Spectrum measured from the native phenolic component (black) overlaid with the spectrum measured from benzoylated phenolic component (gray).

corresponding values were 44.1, 2.30, 2.91, 49.2, and 18%, respectively. The nitrogen content present in both polymers probably originated from the enzymes used in the fractionation. The carbon content of raw suberin is somewhat higher than that of the phenolic component, confirming the presence of long hydrocarbon chains. According to our recent studies (*20*), ca. 30% of the aliphatic suberin monomers are removed from the phenolic component during the fractionation. The difference in the hydrogen contents between the isolated fractions can be considered negligible. Somewhat higher oxygen content in the phenolic component suggests the presence of large numbers of ether bonds or carbonyl groups.

The DSC technique is widely used for measuring, for example, phase transition and glass transition temperatures (T_g) of various polymers. The glass transition temperature is the temperature at which an amorphous solid material such as glass becomes soft on heating or brittle on cooling. The DSC curve of raw suberin (**Figure 2A**) showed two endothermic peaks at approximately 45 and 59 °C, suggesting that the native raw suberin is a mixture of at least two different types of biopolymers. Interestingly, the DSC

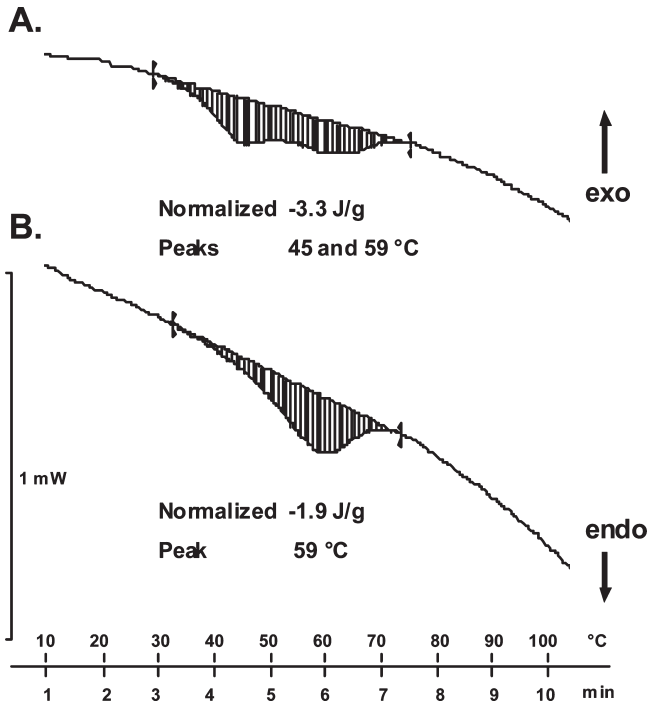

Figure 2. DSC curves measured from (**A**) suberin and (**B**) the phenolic component.

thermogram of the phenolic component (**Figure 2B**) showed only one endothermic peak at 59 °C, that is, exactly at the same position as one of the two peaks of the raw suberin component. It was also notable that in the case of raw suberin nearly 2-fold more heat was required to soften the polymeric structure.

Characterization of Raw Suberin and the Phenolic Component in Solution. Imidazolium-based ionic liquids have been shown to be powerful tools for solubilizing different types of high molecular weight biopolymers under mild reaction conditions (*23*). However, ball-milling of the raw suberin and the phenolic component at rather high temperature (80 °C) using a long incubation time (18 h) was found to be crucial for complete solubilization of the polymers in [Amim]Cl. Once solubilized, all hydroxyl-containing functional groups of the polymers could be phosphitylated with PR[II] reagent, which in turn facilitated ^{31}P NMR measurement of the modified polymers. On the basis of our previous studies with cellulose (*23*), it was assumed that the phosphitylated samples may need further dilution with deuterated chloroform in order to achieve reliable quantitative results and good quality ^{31}P NMR spectra.

The series of ^{31}P NMR spectra measured from ball-milled solubilized raw suberin and the phenolic component in 1, 2, and 3 mL dilutions are shown in **Figures 3** and **4**. The NMR spectra show that raw suberin and the phenolic component contain free functional groups of carbohydrates and of two different phenolics (i.e., guaiacyl- and *p*-hydroxyphenyl structures) because no syringyl structure was detected from the spectra. The peak shape of the carboxylic acid group is rather sharp, suggesting the presence of unbound carboxylic acids rather than the polymerized structure (*24*). The intensity of aliphatic hydroxyl groups somewhat increased as a function of dilution of the samples. The effect of the dilution is also demonstrated in **Figure 5**, which shows the corresponding numerical values of the integrated peak areas in mmol per gram of the sample. Addition of deuterated chloroform strongly affected the solubility and reactivity of the phenolic component with the phosphitylation reagent, although the phase separation was not visually evident in the NMR tube.

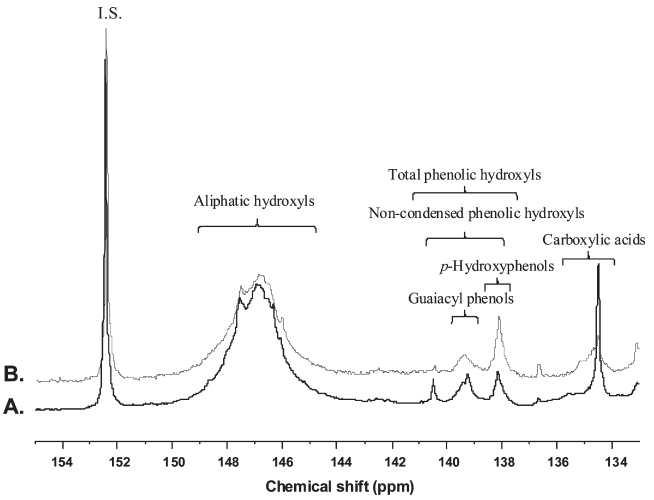

Figure 3. ^{31}P NMR spectra of raw suberin and the phenolic component solubilized in ionic liquid and measured from 1 mL dilutions. The assignments of the various functional groups are shown above the corresponding peaks. I.S. is an internal standard.

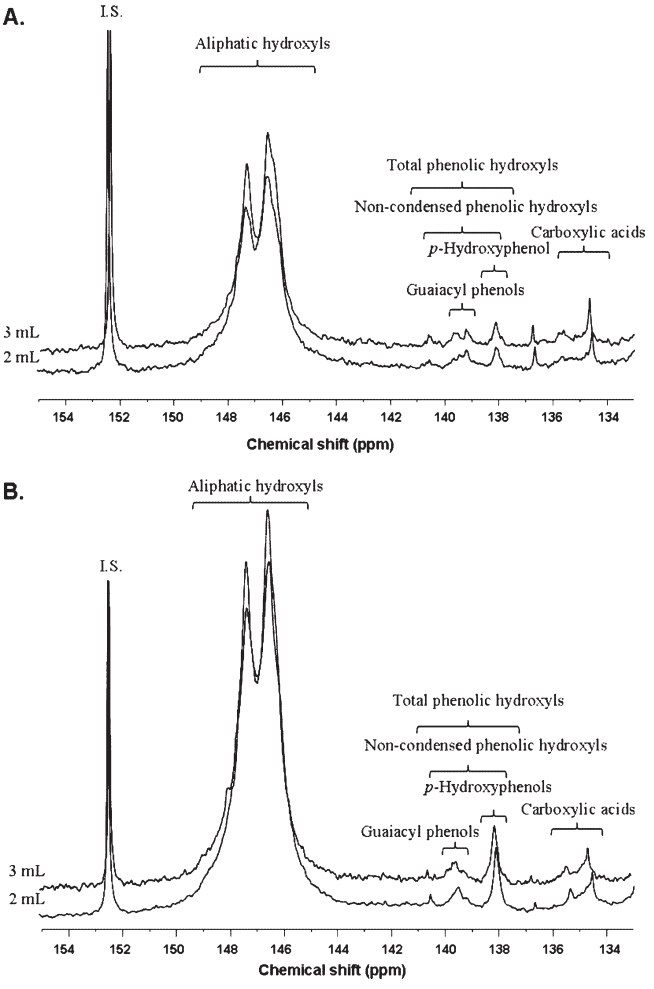

Figure 4. ^{31}P NMR spectra of (**A**) raw suberin and (**B**) the phenolic component solubilized in ionic liquid and measured from 2 and 3 mL dilutions. The assignments of the various functional groups are shown above the corresponding peaks. I.S. is an internal standard.

Dilutions with deuterated chloroform resulted in the formation of a clear liquid, and the intensity of the peaks increased as a function of dilution, showing similar behavior to that of wood

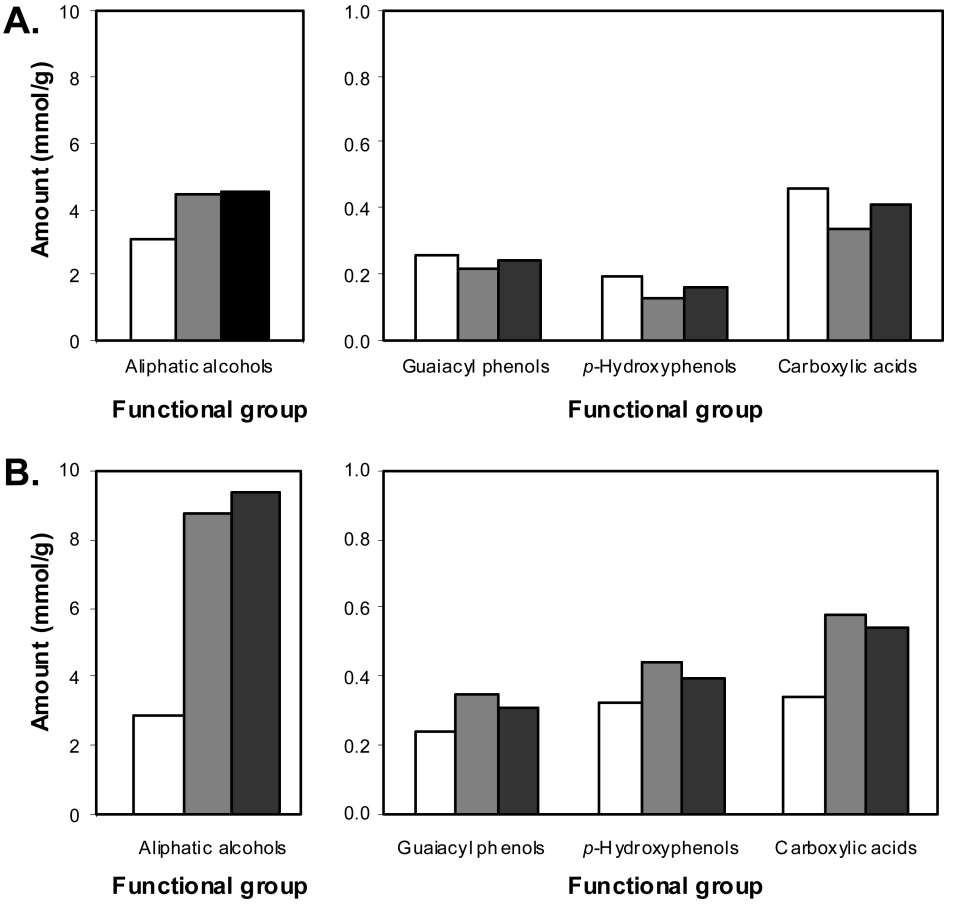

Figure 5. Amounts of various functional groups present in (**A**) raw suberin and (**B**) the phenolic component. Values are calculated from the corresponding ^{31}P NMR spectra shown in **Figures 3** and **4**; 1, 2, and 3 mL dilutions are shown with open, gray, and black filled bars, respectively.

Table 1. Amounts of Different Functional Groups Present in Raw Suberin and in the Phenolic Component

functional group	raw suberin (mmol/g)	phenolic component (mmol/g)
aliphatic alcohols	4.5	9.4
guaiacyl −OH	0.24	0.31
p-hydroxyphenyl −OH	0.16	0.39
carboxylic acids −OH	0.41	0.54

components (*23*). By contrast, in the case of raw suberin, dilution of the sample with deuterated chloroform also resulted in the dissolution of the aggregates, although the effect was not as significant as in the case of the phenolic component.

The numerical values of the amounts of different functional groups as determined from the final points of the dilution series are summarized in **Table 1**. In the case of raw suberin, the amount of aliphatic alcohols, 4.5 mmol/g, is ca. 10 times higher than the sum of phenolic compounds (i.e., 0.40 mmol/g). Unexpectedly, the amount of carboxylic groups, 0.41 mmol/g, was almost the same as the sum of various phenolics present in the polymer. After isolation, the amount of phenolic groups nearly doubled (i.e., up to 0.70 mmol/g). However, the amount of carboxylic acid groups, 0.54 mmol/g, remained almost the same. The somewhat larger amount of aliphatic alcohols, 9.4 mmol/g, present in the phenolic component is most probably due to the exposure of hydroxyl groups after isolation and removal of long chain hydroxy fatty acid and diacid polymers (i.e., aliphatic suberin monomers), which also resulted in a decrease in the carbon content. All of the measurements were performed in duplicate, and the variation between the quantitative determinations was found to be $< 10\%$.

For the GPC analyses, raw suberin and the phenolic component were benzoylated. Yields of the brown benzoylated polymers were 150 and 175%, respectively, showing extensive derivatization of the biopolymers. Before the GPC analyses, the success of benzoylation was further confirmed by FTIR spectroscopy. The spectra measured from the untreated raw suberin and from the phenolic component as well as from the benzoylated polymers are overlaid in **Figure 1B**,**C**, respectively. In both cases, two intense bands of an ester bond at ca. 1725 and 1270 cm^{-1} could be detected from the spectra, indicating a high degree of benzoylation of the polymers. The GPC profiles of the modified raw suberin and phenolic components are shown in **Figure 6**. Rather well-defined Gaussian distribution bands were obtained for both polymers. The molecular weights determined from the corresponding chromatograms of modified raw suberin and the phenolic component were 44×10^3 and 27×10^3 g/mol, respectively. In addition to the main components, some lower MW polymers could be detected from the chromatograms.

Functional Group Comparison of Raw Suberin and the Phenolic Component. Enzymatic fractionation and extraction of soluble components of suberin followed by methanolysis for detailed structural characterization of the phenolic component revealed that aliphatic hydrocarbon chains were completely truncated from the raw suberin because no band for the ester linkages of aliphatic fatty and diacid polymers and linkages to aromatic components was detected from the FTIR spectrum of the isolated phenolic component. The carbon contents of the polymers, as detected by the element analysis, also confirm the conclusion that suberin contains long aliphatic double-bonded chains forming the hydroxy fatty acid and diacid polyester. However, after

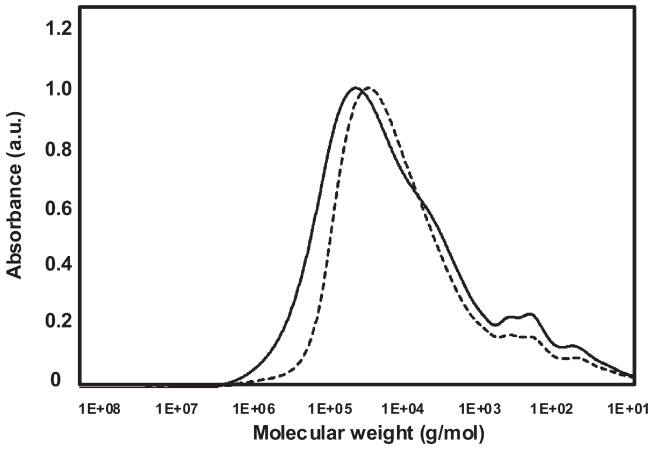

Figure 6. GPC profiles of benzoylated raw suberin (solid) and phenolic component (dashed).

methanolysis, the amount of the cellulosics in the phenolic component was still very high, as could be seen from the intense ether band. This is not surprising since it is well-known that suberin is linked to plant cell walls via carbohydrates (*1*, *4*, *16*, *25*, *26*). The presence of representative bands for aromatics verified that the structure of the phenolic component of potato suberin is a mixture of cellulose and aromatics when using the isolation procedure described.

Detailed quantitative structural analysis of raw suberin and the phenolic component by ^{31}P NMR spectroscopy confirmed that the amount of carbohydrates was remarkably high in both polymers, as the amount of aliphatic alcohols was over 10 times higher than the sum of phenolics (i.e., the sum of guaiacyl- and *p*-hydroxyphenyl groups). However, when the amount of aliphatic alcohols quantified as mmol per gram was converted to cellulose as gram per gram, it became obvious that not all of the aliphatic hydroxyl groups are derived from carbohydrates. This is not surprising because the phenolic component is assumed to be rather similar to lignin and thus may have hydroxyl groups at least to some extent in α, ss, and γ positions of repeating hydroxypropyl units of the polymer. When the phenolic component was diluted from 1 to 3 mL volume, a remarkable increase in the peak intensity of the aliphatic alcohols was observed. However, the increase in phenolic and carboxylic acid groups was less significant. It is worth mentioning that similar behavior has been observed earlier in our studies with various wood samples (*23*). Clearly, in the case of raw suberin, the effect of dilution was less important. After methanolysis of raw suberin, the amount of phenolic groups increased from 0.40 to 0.70 mmol/g. The decrease in molecular weight after chemical treatment (from 44×10^3 to 27×10^3 g/mol) is also in good agreement with removal of polymeric long chain acid moieties.

DSC data measured from the isolated component support the conclusions presented above. In the case of raw suberin, two well-resolved bands were detected, but in the case of the phenolic component, only a single broad band was observed. The maximum band intensities of the DSC curves are located at low temperatures (i.e., below 100 °C), as expected on the basis of studies with cork suberin (*16*), also suggesting strong water binding properties. For example, in the case of dry cellulose, T_g is between 220 and 250 °C (*26*), but in the case of lignin, T_g values up to 550 have been reported (*27*, *28*). Softeners such as water affect the thermal behavior of biopolymers. It has been shown that even small amounts of absorbed water cause a pronounced shift of T_g to lower temperatures due to the breakdown of hydrogen bond networks and reduction of the cohesive energy between the polymer chains. In general, hydrophilic cellulosics may absorb large amounts of water, but hydrophobic phenolic lignin may bind only a limited amount of water. Various small molecules, such as glycerol, present in the sample can also lower the T_g value of the polymer. The difference in thermal behaviors of raw suberin and the phenolic component clearly points toward success of the fractionation procedure because in the case of the phenolic component only one clear band was detected.

In our recent studies using ^{13}C CP MAS NMR (*20*), it was shown that the phenolic component of potato raw suberin contains mainly carbohydrates and lignin-like aromatics. Signals for carboxyl groups derived from aromatics and polysaccharides could be identified from the ^{13}C CP MAS NMR spectrum. Those results are compatible with the data presented in this paper. In the ^{13}C NMR studies of Bernards et al. (*24*) and Stark et al. (*29*) with wound-induced suberized potato periderm, the nature of the aromatic part of polyphenolic−polysaccharide material was also proposed to be a lignin-like ferulic-acid-based polymer. In the case of cork suberin, polysaccharide residual material has also been extensively studied by various analytical methods in the solid state (*24*, *29*−*31*). The phenolic component was found to be, at least partially, a true guaiacyl lignin (*30*). The polysaccharides were composed of approximately equal proportions of cellulose and hemicelluloses (*31*).

Functional Group Comparison of the Phenolic Component to That of Lignin. Studies with potato raw suberin indicate that the phenolic component also contains large amounts of carbohydrates, most probably celluloses, suggesting that it is attached to the plant cell wall via ether linkages. Thus, it is not surprising that the phenolic component of suberin has been difficult to isolate in the native state, which has hampered its structural characterization (*1*, *4*, *25*, *26*). This means that the isolated phenolic components of suberin and lignins will always contain rather large amounts of carbohydrate as impurity, regardless of the method of fractionation. Although mechanical, chemical, and enzymatic isolation of the phenolic component of suberin from different parts of the plants, as in the case of lignin from wood, definitely alters the structure of the biopolymers, it is still possible that the smallest repeating units of isolated suberin and lignin have the same morphology as the polymers have in the intact cell walls. Evidently, isolation of the phenolic component of suberin or of lignin without damaging the structure is still a major challenge in biopolymer chemistry.

The functional properties of the phenolic components of suberin and lignin result from their chemical structure and from the isolation procedure of the biopolymers. However, a fractionation method which yields an unchanged form of the biopolymer followed by complete structural characterization has not yet been established. Solid-state ^{13}C NMR has been shown to provide quantitative analysis of some of the key structural features of lignin in plant tissues as well as in isolated lignins (*32*). The technique has been successfully used also in suberin analyses (*11*, *14*, *15*, *20*). However, interpretation of the ^{13}C NMR spectra depends completely on the availability of the model compounds. Although the method allows nondestructive characterization of the macromolecule, the technique is hindered by the difficulty in making unequivocal assignments due to the extensive overlapping of phenolic and carbohydrate resonances. In addition, it suffers from low sensitivity and the need for a large quantity of dry material. On the basis of the results obtained from raw suberin and phenolic components dissolved in ionic liquid followed by ^{31}P NMR measurement, the protocol is proposed as a general methodology to characterize the chemical structures of highly insoluble biopolymers. Further derivatization of the polymers in ionic liquids followed by GPC enables estimation of the molecular weights of the polymers. Ball-milling of the polymers

was crucial in order to obtain a homogeneous solution for structural characterization of the polymers. By including the ball-milling step in the isolation protocol already before enzymatic treatments, the carbohydrate content of both of the isolated components might have been remarkably decreased because the reactivity of the enzymes toward smaller particles is much higher than toward large particles.

Finally, it can be summarized that in the enzymatically isolated extractive free raw suberin the amounts of various functional groups included the following: aliphatic alcohols 4.5 mmol/g, phenolics 0.40 mmol/g, and carboxylic acids 0.41 mmol/g. The corresponding results for the phenolic component were 9.4, 0.70, and 0.54 mmol/g, respectively. Thus, the two-domain structural model of suberin proposed by Bernards et al. (*1*, *9*, *24*) is strongly supported by the data presented in this paper. The phenolic part of potato suberin contained guaiacyl- and *p*-hydroxyphenyl structures. Thus, the isolated phenolic component from plant origin may be a valuable source of antioxidative compounds for food, cosmetics, and medical applications, motivating further development of the isolation protocol. Structural characterization of the phenolic component at the functional group level will also have a major impact on understanding of the biosynthesis of suberin.

ABBREVIATIONS

[Amim]Cl, 1-allyl-3-methylimidazolium chloride; CP MAS, cross-polarization magic angle spinning; Cr[acac]$_3$, chromium-(III)acetylacetonate; DSC, differential scanning calorimetry; GPC, gel permeation chromatography; PR[II], 2-chloro-4,4,5,5-tetramethyl-1,3,2-dioxaphospholane; T_g, glass transition temperature.

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