Chemoenzymatic Fractionation and Characterization of Pretreated Birch Outer Bark

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ABSTRACT: In this study, the application of different chemical and enzymatic treatment methods for the fractionation of the birch outer bark components was evaluated. More specifically, untreated and steam exploded, hydrothermally and organosolv treated bark samples were incubated with enzyme mixtures that consisted of cellulases, hemicellulases and esterases, and the effect of enzymes was analyzed with ^{31}P NMR and $\{^{13}\text{C}-^1\text{H}\}$ HSQC. The biocatalysts performed the cleavage of ester bonds resulting in reduction of methoxy and aliphatic groups in the remaining solid fraction, whereas the aromatic fraction remained intact. Moreover, the suberin and lignin fraction were isolated chemically and their properties were characterized by gas chromatography (GC–MS), ^{31}P NMR, $\{^{13}\text{C}-^1\text{H}\}$ HSQC and gel permeation chromatography (GPC). It was demonstrated that the lignin fraction was enriched in guaiacyl phenolics but still contained some associated aliphatic acids and carbohydrates, whereas the suberin fraction presented a polymodal pattern of structures with different molecular weight distributions. This work will help in getting a deeper fundamental knowledge of the bark structure, the intermolecular connection between lignin and suberin fractions, as well as the potential use of enzymes in order to degrade the recalcitrant bark structure toward its valorization.

KEYWORDS: *Betula pendula*, Outer bark, Suberin structure, Cutinase, ^{31}P NMR, $\{^{13}\text{C}-^1\text{H}\}$ HSQC

INTRODUCTION

The exploitation of lignocellulosic biomass for its effective valorization and the extraction of compounds that can be used for the production of polymers should be closely related to the woody feedstocks available in large quantities in timber/lumber industries across the EU. The major biomass raw material in Northern Europe comes from conifers, providing about 45% of the world's annual timber production (FAO 2006). In Sweden, around 70% of the country's area is covered by forests accounting for around 28 million hectares. Forest products deliver half of the net national income, so conifers are of great economic importance in these areas. According to The Swedish National Chemical defense, silver birch (*Betula pendula*) is one of the major tree species in the country as it is the third most abundant after spruce and pine and it constitutes the dominant tree species in plywood-making as well as for pulpwood and fuel. The total production of market pulp in Sweden amounts to approximately 3.8 million tons annually, according to The Swedish Forest Industries Fact and Figures, leading to the production of considerable amounts of birch bark as a residual product from log debarking, usually burned for energy production. The bark composes 2–3.4% of the total mass of the birch log and has been the subject of intensive research because of its high content of compounds with wide beneficial

chemistry and bioactivity, such as pentacyclic lupine-type triterpenes and suberinic polyesters.^{1,2}

Suberin is a lipid-derived insoluble polyester mainly found in the periderm of plants, such as tree barks and tuber skins, but also in a number of other plant tissues, including the epidermis and hypodermis of roots, the endodermis.³ This hydrophobic polymeric material is deposited in the secondary cell wall of internal and peripheral dermal tissues during cell wall differentiation or as a response to stress and wounding,⁴ thus creating an apoplastic barrier that controls water, gas and ion flow and protects the plant against pathogens. Suberin is composed of two covalently linked domains, a polyaliphatic domain composed of hydroxy or epoxy fatty acids joined by ester linkages and polyphenolic domain formed by hydroxycinnamic acids and their derivatives, impregnated in the inner side of the primary cell wall.^{5–7} Intermonomer ester bonds between fatty acids, ester/ether cross-linkages between fatty acids and hydroxycinnamates, as well as C–C, amide and ether bonds of

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Table 1. Summary of All the Pretreatment Conditions, the Calculated Severity Factor/Combined Severity (SF/CS) and % Dissolved Bark

batch	type	ethanol/water	T (°C)	t (min)	bark solubilization (%)	SF/CS
#1	HT		200	10	12.00	3.94
#2	SE		195	10	23.33	3.97
#3	OS/SE	10/90	203	60	21.67	4.81
#4	OS 50:50	50/50	195	60	21.00	4.81
#5	OS 80:20	80/20	160	240	26.88	4.15

Figure 1. Flow diagram of the experimental steps followed.

hydroxycinnamates to other cell wall components create a complex and rigid network.^{8–10} This may justify why suberin is difficult, perhaps even impossible, to isolate in its pure form and in its native state, which has hampered its structural characterization.¹¹ The aliphatic polyester domain is composed mostly of long chains (C₁₆–C₂₆) of alkanols, alkanoid acids, ω -hydroxyalkanoic acids, α,ω -alkanedioic acids and glycerol.^{8,11,12} Many of these classes of molecules can be used as starting materials in the synthesis and production of different poly- and oligomeric value-added products, such as polyols, and polyurethanes.^{13,14}

Not all available biomass is immediately accessible in a form suitable for direct valorization. The application of an initial pretreatment that will convert raw materials in a first step to a form more amenable to a second step such as an enzymatic degradation is an integral key element in all the biotechnological technologies employed for the exploitation and valorization of lignocellulosic biomass.¹⁵ In this study, *B. pendula* outer bark was thus treated hydrothermally, using steam explosion and organosolv pretreatment, and was subsequently subjected to enzymatic hydrolysis with celluloses/hemicelluloses for removal of polysaccharides followed by treatment with cutinases. The results obtained for the pretreated samples were additionally compared to those obtained for untreated *B. pendula* outer bark after identical biotechnological treatment. The enzymes were chosen according to their specific activities; cutinases (EC 3.1.1.74) can hydrolyze natural cuticular polyesters (cutin, suberin) to lower molecular weight compounds.^{16–18} Their potential use can provide an appealing alternative to chemical depolymerization processes, as the latter do not offer selectivity and lead to loss of different functionalities (epoxy, hydroxyl and carboxylic).¹⁹ Esterases that have been identified in the secretome of various fungi species that naturally colonize cork cell walls in the presence of suberin presumably release long chain fatty acids with hydroxyl or epoxy moieties.^{20,21}

In the present study, suberin and lignin fractions from untreated and pretreated bark samples were chemically isolated and characterized. $\{^{13}\text{C}-^1\text{H}\}$ -HSQC and quantitative ^{31}P NMR measurements²² were used for structural analysis and quantitative determination of various hydroxyl groups, respectively, such as alcohols, phenolics, and carboxylic acids present in all fractions. Gel permeation chromatography (GPC) and gas chromatography coupled with mass spectrometry (GC–MS) were used to determine the molecular weights of the isolated polymers and the monomeric composition of suberinic material, respectively. The results shed light on the effect of different pretreatment methods on the structural conformation of different cell wall constituents of birch outer bark and the properties of the solid fraction after enzymatic treatment with esterases. This work will contribute to a better understanding of the cross-linking of the lignin–suberin polymeric matrix, in order to improve suberin extraction processes and bark valorization.

MATERIALS AND METHODS

The bark from the European hardwood *Betula pendula*, obtained as residual byproduct of commercial debarking in the pulp mill Smurfit Kappa, Sweden, was ground in a knife mill (Retsch SM 3000) using an output sieve of 1 mm × 1 mm. The bark was submitted to different pretreatment methods, including hydrothermal (HT), steam explosion (SE) and organosolv (OS) pretreatment, as summarized in Table 1 and described in our previous study.²³ Extractives were fully removed from the solid fractions obtained after pretreatment by successive Soxhlet extractions with dichloromethane, ethanol and water. The extractive-free bark sample was ball-milled at 300 rpm for 12 h (Retsch S100) and subjected to polysaccharide removal with the combination of celluloses, hemicelluloses, and oxidative enzymes, as described below. The extractive/polysaccharide-free fraction was then treated with a polyesterase enzyme mixture. This fraction was also subjected to acidolysis for lignin extraction and alkaline methanolysis for suberin isolation. Lignin fractions were analyzed using gel permeation chromatography (GPC), ^{31}P NMR and $\{^{13}\text{C}-^1\text{H}\}$ HSQC measurements. Suberin esters were analyzed with GPC, quantitative ^{31}P NMR and $\{^{13}\text{C}-^1\text{H}\}$ HSQC measurements, whereas the composition of

Figure 2. 2D-NMR HSQC spectra of untreated and pretreated *Betula pendula* outer bark. (A) untreated bark, (B) hydrothermally pretreated bark, (C) steam exploded bark, (D) organosolv pretreated SE/OS 10:90 bark, (E) organosolv pretreated OS 50:50 bark and (F) organosolv pretreated OS 80:20 bark. See Table 3 for signal assignments (purple, methyl groups; yellow/green, methylene groups; light blue, acetate groups; red, methoxy groups; blue, β -O-4' related structures; gray, sugars; brown, aromatic signals).

monomers was identified with gas chromatography coupled with mass spectrometry (GC–MS). Bark solid fractions before and after enzymatic pretreatment were analyzed by quantitative ^{31}P NMR and $\{^{13}\text{C}-^1\text{H}\}$ HSQC measurements. The analytical protocol applied in this work is illustrated in Figure 1.

Removal of Polysaccharides. Treatment with a cellulase/xylanase enzyme mixture occurred at a reaction volume of 100 mL and the substrate initial concentration was 5% (w/v) dry matter. Reactions took place for 12 h at 50 °C, pH 5.0 (phosphate–citrate buffer, 100 mM). The enzymes that were used included the cellulase

cocktail CTec2 (Novozymes, 20 FPU/g substrate), one xyloglucanase (XG, Megazyme, 0.25 mg/g substrate) and one xylanase (Xyl6, offered from Dyadic, 0.25 mg/g substrate), as well as one β -glucosidase (*MtBGL3*) and one lytic polysaccharide monooxygenase (LPMO, *MtGH61*) from *Myceliophthora thermophila*, both heterologously expressed and produced in *Pichia pastoris*.^{24,25} LPMO was added at a loading of 0.1 mg/g substrate and β -glucosidase was added in excess in order to prevent inhibition caused by the cellobiose produced. After the enzymatic reaction, the remaining solid fraction was washed extensively prior to any further analysis.

Table 2. Compositional Analysis of Bark Samples after Extractives Removal^a

	untreated	HT	SE	OS/SE	OS 50:50	OS 80:20
total sugars	7.34	7.47	8.20	8.75	8.75	9.76
ash	1.11	0.57	0.38	0.27	0.27	0.30
suberin	72.69	56.19	56.02	50.37	59.36	44.44
lignin	15.03	29.12	29.00	29.98	23.12	32.92

^aMaterials and methods used for this analysis are described in our previous study.²³

Table 3. Assignments of ¹³C–¹H Correlation Peaks in the 2D-NMR HSQC spectra of *Betula pendula* Outer Bark and the Derived Samples after Pretreatment and Enzymatic Hydrolysis

	δ ¹ H	δ ¹³ C	assignment
aliphatic region	0.8–1.06	13.2–18.7	C–H in aliphatic methylic groups (–CH ₃)
	2.01	20.6	C–H in acetate groups (C ₂ H ₃ O ₂ –)
	1.22–1.57	24.3–28.5	C–H in aliphatic methylenic groups (–CH ₂ –)
	2.26	33.2	C–H in methylenes linked to carboxylic moieties (CH ₂ COO; CH ₂ COOH)
side-chain region	3.74	55.4	C–H in methoxy groups (–O–CH ₃ , –O–CH ₂ –)
	3.57	60.8	C _γ –H _γ in γ-hydroxylated β-O-4' substructures (A)
	3.44	60.3	C _γ –H _γ in γ-acylated β-O-4' substructures (A')
	4.5	73.1	C _α –H _α in β-O-4' substructures (A) linked to a G-unit
aromatic region	6.65	103.5	C ₂ –H ₂ and C ₆ –H ₆ in etherified syringyl units (S)
	6.99	110.9	C ₂ –H ₂ in guaiacyl units (G)
	6.67 and 6.77	114.6	C ₅ –H ₅ and C ₆ –H ₆ in guaiacyl units (G)
	7.2	128.0	C _{2,6} –H _{2,6} in p-hydroxyphenyl units (H)

Treatment with Esterases. Polyestrase mixture was composed of feruloyl esterase (MtFAE1) and glucuronoyl esterase (MtGE) from *M. thermophila* that were heterologously expressed and produced in *P. pastoris*,^{26,27} as well as cutinases (Axe1 and Axe2, offered from Dyadic). Reactions took place at 40 °C, pH 6.0 (phosphate–citrate buffer, 100 mM), final volume of 20 mL, and the substrate initial concentration was 5% (w/v) dry matter. Enzyme loading for MtFAE1 and MtGE was 1 mg/g substrate, whereas cutinases were added to a concentration of 0.5 mg/g substrate. After treatment with esterases, the remaining solid fraction was washed extensively prior to any further analysis.

Lignin Isolation. 3 g of extractive-free, cellulase/xylanase treated material was suspended in 50 mL of acidified dioxane–water (85:15 w/w) solution. This mixture was then refluxed (boiling point 86 °C) under nitrogen for 4 h. The resulting solution was filtered and neutralized with sodium bicarbonate. The neutralized solution was added dropwise to 500 mL of acidified deionized water (pH 2.0). The precipitated lignin was isolated by centrifugation, washed and freeze-dried.

Suberin Isolation. A 1.5 g sample of extractive-free, cellulase/xylanase treated material was refluxed with 100 mL of a 3% methanolic solution of NaOCH₃ in CH₃OH for 3 h. The sample was filtrated and washed with methanol; the residue was refluxed with 100 mL of CH₃OH for 15 min and filtrated again. The combined filtrates were acidified to pH 6 with 2 M H₂SO₄ and evaporated to dryness. The residues were suspended in 50 mL of water and the alcoholysis products recovered with dichloromethane in three successive 50 mL dichloromethane extractions. The combined extracts were dried over anhydrous Na₂SO₄, and the solvent was evaporated to dryness.

2D-NMR HSQC Analysis. NMR samples were prepared as follows: 60–70 mg of ball-milled dry bark/suberin sample was added to 600 μL of DMSO-*d*₆ solution of chromium(III) acetylacetonate (57.2 mM) and then placed in an ultrasonic bath and sonicated for 1 h to homogenize the NMR sample. The resulting gel was transferred directly into a 5 mm NMR tube. HSQC spectra were recorded at 27 °C on a Bruker 700 MHz instrument equipped with TopSpin 2.1 software. Spectra were referenced to the residual signals of DMSO-*d*₆ (2.49 ppm for ¹H and 39.5 ppm for ¹³C spectra). {¹³C–¹H} HSQC spectra were obtained after using the standard Bruker pulse program (hsqcetpsisp2) with the following parameters for acquisition: TD = 2048 (F2), 512 (F1); SW = 13.0327 ppm (F2), 160 ppm (F1); O1 = 4200.54 Hz; O2 = 14083.02 Hz; D1 = 2 s; CNST2 = 145; acquisition time F2 channel = 112.34 ms; F1 channel = 8.7102 ms. NMR data were processed with MestreNova (Version 8.1.1, Mestrelab Research) by using a 60°-shifted square sine-bell apodization window; after Fourier transformation and phase correction a baseline correction was applied in both dimensions. The central DMSO solvent peak was used as internal reference (δ _C 39.5, δ _H 2.5 ppm). 2D-NMR cross-signals were assigned as in previous studies.^{28–34}

³¹P NMR Analysis. Approximately 30 mg of sample was transferred into an NMR tube, dissolved in 400 μL of pyridine/deuterated chloroform (1.6:1 (v/v)). 100 μL of phosphitylating reagent I (2-chloro-1,3,2-dioxaphospholane, 95%, Sigma-Aldrich) or reagent II (2-chloro-4,4,5,5-tetramethyl-1,3,2-dioxaphospholane, 95%, Sigma-Aldrich) were added and 5-norbornene-2,3-dicarboximide or cholesterol were used as internal standards with reagent I and II, respectively. Chromium(III) acetylacetonate was used as the relaxation agent. 100 μL of the solution prepared from 0.1 mmol/mL of internal standard and 0.0143 mmol/mL of relaxation agent were added. The mixture was then incubated at room temperature for 1 h (reagent I) or 2 h (reagent II) under continuous stirring. The spectra were acquired using a Bruker 300 MHz spectrophotometer (256 scans at 20 °C) equipped with a Quad probe dedicated to ³¹P, ¹³C, ¹⁹F and ¹H acquisition. All chemical shifts reported are relative to the reaction product of water with the phosphitylating reagent I or II, which gives a sharp signal in pyridine/CDCl₃ at 121.10 or 132.2 ppm, respectively. Quantitative analysis was performed based on previous literature reports.³⁵

Gel Permeation Chromatography (GPC) Analysis. Approximately 5 mg of each sample was suspended in 1 mL glacial acetic acid/acetyl bromide (9:1 v/v) for 2 h. The solvent was then carefully fully removed *in vacuo*, and the residue was dissolved in the solvent or solvent system of choice and filtered over 0.45 μm syringe filter prior to injection. By means of a sample loop, aliquots of 20 μL of the filtered “sample”-solutions were analyzed at a time. GPC analyses were performed using a Shimadzu Analytical HPLC instrument consisting of a controller unit (CBM-20A), a pumping unit (LC 20AT), a degasser (DGPU-20A3), a column oven (CTO-20AC), a diode array detector (SPD-M20A), and a refractive index detector (RID-10A), and controlled by Shimadzu LabSolutions (Version 5.42 SP3). A setup comprising three analytical GPC columns (each 7.5 × 30 mm) in series were realized for analyses: Agilent PLgel 5 μm 10000 Å, followed by Agilent PLgel 5 μm 1000 Å, followed by an Agilent PLgel 5 μm 500 Å. HPLC-grade THF (Chromasolv, Sigma-Aldrich) was used as eluent (0.75 mL min^{−1}, at 40 °C). Standard calibration was performed with polystyrene standards (Sigma-Aldrich, MW range 162–5 × 10⁶ g mol^{−1}). Final analyses of each sample was performed using the intensities of the UV signal at λ = 280 nm, employing a tailor-made MS-Excel-based table calculation as outlined elsewhere.³⁶

Gas Chromatography (GC–MS) Analysis. A known amount (1–2 mg) of sample was dissolved in 500 μL chloroform/50 μL pyridine and components containing hydroxyl groups were converted into their trimethylsilyl (TMS) ethers by adding 150 μL of bis(trimethylsilyl)trifluoroacetamide and 50 μL of trimethylchlorosilane. 50 μL acetovanillin 25 mM were added as internal standard. After the mixture had stood at 70 °C for 30 min under continuous stirring, the methyl esters/trimethylsilyl (TMS) ethers were immediately analyzed. Analysis was done on 5 μL aliquots using a Shimadzu GCMS QP2010 Ultra equipped with an AOI20 autosampler unit. A SLB-5 ms Capillary GC Column (L × I.D. 30 m × 0.32 mm, df 0.50 μm) was

Figure 3. 2D-NMR HSQC spectra of *Betula pendula* outer bark after enzymatic treatment with cellulases, hemicellulases and esterases. (A) Untreated bark (not subjected to any pretreatment prior to incubation with enzymes), (B) hydrothermally pretreated bark, (C) steam exploded bark and (D) organosolv pretreated OS 80:20 bark. All organosolv pretreated bark samples showed identical signals after enzymatic treatment, thus only one is shown and is considered to be representative. See Table 3 for signal assignments (purple, methyl groups; yellow/green, methylene groups; red, methoxy groups; blue, β -O-4' related structures; gray, sugars).

used as stationary phase, ultrapure helium as the mobile phase, at 100 kPa pressure, 240 °C injection temperature, 200 °C interface temperature, using the following temperature program: 50 °C start temperature for 1 min, 10 °C min⁻¹ heating rate, 240 °C final temperature for 15 min; electron ionization was realized using ca. 85 eV. Analysis was done using Shimadzu LabSolutions GCMS Solution software (Version 2.61). The various components were identified by comparing their mass spectra with those from NIST11 library, by a specific examination of their characteristic fragmentation patterns and with previously published data. The relative abundance of the compounds (relative with respect to the ionisability of each species under analysis conditions) was calculated from the peak areas in the total ion gas chromatogram.

For estimating the artifacts caused by the necessary ionization prior to detection, GC-FID analyses were performed in parallel on the same GC instrument controlled by the same software using a second injection port leading to a SLB-5 ms Capillary GC Column (L $\times$ I.D. Thirty m $\times$ 0.32 mm, df 0.50 μ m) as stationary phase. Hydrogen (produced in a CLAlND HyGEN200 hydrogen generator) was used as carrier gas and to feed the flame ionization detector (FID) together with compressed air under otherwise identical analysis conditions; Helium was used as makeup gas.

RESULTS AND DISCUSSION

¹³C–¹H} HSQC and ³¹P NMR Analysis before/after Enzymatic Treatment. The HSQC spectrum of untreated bark composed of 72% suberin, 17% lignin and 10% polysaccharides (Table 2) is presented in Figure 2A. In the aliphatic region, the dominant presence of a major group of signals associated with suberin methylene and methyl groups is observed (δ_C/δ_H 24.3–33.2/1.22–2.26 and δ_C/δ_H 13.2–18.7/0.8–1.06). The side-chain region of the spectra gives useful information about the different interunit linkages present in the lignin and suberin moieties (Table 3). In this region, cross-signals from methoxy groups (δ_C/δ_H 55.4/3.74) and side-chains in β -O-4'-substructures are the most prominent. The C_γ – H_γ correlations in hydroxylated β -O-4'-substructures are seen at δ_C/δ_H 60.8/3.57 and 60.6/3.44, whereas the signals at 63.2/3.98 from C_γ – H_γ correlations of γ -acylated units show the presence of acylation of lignin at the γ -carbon of the side-chain which is common in the case of other lignins and has also been observed in cork HSQC spectrum.^{18,37,38} Strong signals for acetate groups were also observed in the HSQC spectrum of untreated bark at δ_C/δ_H 20.6/2.01, indicating that acetates might be the acylating group on the γ -OH of this lignin. Signals from hydroxylated groups are more intense than those from

Figure 4. (A) ^{31}P NMR spectrum of steam exploded extractive-free *Betula pendula* outer bark before and after enzymatic treatment using 2-chloro-4,4,5,5-tetramethyl-1,3,2-dioxaphospholane as phosphitylating agent; IS, internal standard (5-norbornene-2,3-dicarboximide). (B) Content of estimated aliphatic hydroxyls in bark samples before and after enzymatic treatment.

acylated. The $\text{C}_\alpha\text{--H}_\alpha$ correlations in $\beta\text{-O-4'}$ -substructures are seen in low amounts at $\delta_\text{C}/\delta_\text{H}$ 73.1/4.5. The main cross-signals in the aromatic regions of the HSQC spectrum of untreated bark correspond to the different lignin units. The G units show different correlations for $\text{C}_2\text{--H}_2$ ($\delta_\text{C}/\delta_\text{H}$ 110.9/6.99), and for $\text{C}_5\text{--H}_5$ ($\delta_\text{C}/\delta_\text{H}$ 114.6/6.67 and 6.77). The signals for $\text{C}_{2,6}\text{--H}_{2,6}$ of S and H units, detectable in traces, show a signal for the $\text{C}_{2,6}\text{--H}_{2,6}$ correlation at $\delta_\text{C}/\delta_\text{H}$ 103.5/6.65 and $\delta_\text{C}/\delta_\text{H}$ 128/7.02 respectively.

In the spectrum of steam exploded bark (Figure 2C), signals in the aliphatic region are most prominent, whereas signals within the range of $\delta_\text{C}/\delta_\text{H}$ 76.8–79.9/3.61–3.75 and 106.9–109.3/4.66–4.48 corresponding to polysaccharides (mainly xylan) disappear. In organosolv treated bark (Figure 2D–F), correlations from aliphatic methylic groups (--CH_3) ($\delta_\text{C}/\delta_\text{H}$ 13.2–18.7/0.8–1.06) appear more intense and resolved than in the untreated bark, whereas signals in the aromatic region are completely absent. Signals from hydroxylated $\beta\text{-O-4'}$ -substructures appear in low amounts compared to signals from acylated $\beta\text{-O-4'}$ -substructures that remain intense. Organosolv pretreatment typically results in more than 50% lignin removal through cleavage of lignin-carbohydrate bonds and $\beta\text{-O-4'}$ interunit linkages and subsequent solubilization in the organic solvent. El Hage et al.³⁹ suggested that the cleavage of $\beta\text{-O-4'}$ linkages is the major mechanism of lignin breakdown during organosolv pretreatment of *Miscanthus giganteus*. In the case of bark, it seems that acylated groups are cleaved in low extent in organosolv processes. Cleavage of bonds in the polysaccharidic

Figure 5. (A) 2D-NMR HSQC spectra of suberin structural components isolated with alkaline methanolysis from untreated *Betula pendula* outer bark. (B) Superimpose of suberin isolated from untreated (gray) and steam exploded (yellow-red) outer bark. See Table 3 for signal assignments (purple, methyl groups; yellow/green, methylene groups; red, methoxy groups; light blue, primary/secondary alcohols; brown, aromatic signals).

part of outer bark cell walls is profound in spectra of all organosolv treated materials, as correlations for (1–4) linked $\beta\text{-D-xylopyranoside}$ units and signals in the polysaccharide anomeric region are not observed.

After enzymatic treatment with cellulases, hemicellulases and esterases, all bark samples showed higher solubility in the $\text{DMSO-}d_6$ after the enzymatic treatment giving more homogeneous gels for analysis; the best results, however, were obtained with steam exploded bark. All spectra show broader signals, which are consistent with the partial collapse of cellular structure upon the enzymatic treatment, leading to a more amorphous and disorganized structure. Correlations from polysaccharides in the side-chain region of spectra almost disappear after the synergistic action of cellulases and hemicellulases; the same is observed for the acetate groups (Figure 3A–D). Signals assignable to methoxy groups and $\beta\text{-O-4'}$ -substructures are less intense in steam exploded and hydrothermally treated bark when compared to nonenzymatically treated materials, indicating the cleavage of ester bonds by cutinases. These signals are absent in all organosolv treated samples. Signals in the aliphatic region remain clearly visible after the action of enzymes, whereas methyl groups and methylenes adjacent to polysaccharides are comparatively less

Table 4. Assignments of ^{13}C – ^1H Correlation Peaks in the 2D-NMR HSQC Spectra of Suberin Isolated from *Betula pendula* Outer Bark

	$\delta^1\text{H}$	$\delta^{13}\text{C}$	
aliphatic region	0.6–0.95	15–15.5	C–H in aliphatic methyl groups ($-\text{CH}_3$)
	1.97	26.3	C–H in allylic groups ($\text{CH}_2=\text{CH}-\text{CH}$)
	1.22–1.30	28.4–32.2	C–H in aliphatic methylenic groups ($-\text{CH}_2-$)
	1.41–1.52	32.2–32.5	C–H in methylenes in the β -position to hydroxylic, ester and carboxylic groups ($-\text{CH}_2\text{CH}_2\text{CO}$; $-\text{CH}_2\text{CH}_2\text{O}$)
	2.11–2.43	33.1	C–H in methylenes linked to carboxylic moieties (CH_2COO ; CH_2COOH)
	1.33	37.5	C–H in $-\text{CH}_2-\text{CH}(\text{OH})-$ groups
side-chain region	3.61	51.2	C–H in methoxy groups ($\text{CH}_3-\text{O}-(\text{C}=\text{O})-$)
	3.83	55.5	C–H in methylenes adjacent to ester groups ($\text{O}-\text{CH}_2$)
	2.82	56.7	C–H in epoxide methynes (C_9-C_{10}) of $\text{C}_{18-9,10}$ epoxyacids
	3.4	60.5	C–H in primary alcohols (CH_2-OH)
	3.98	63.35	C–H in methoxy groups ($-\text{CH}_2-\text{O}-(\text{C}=\text{O})-$)
	3.18	73.1	C–H in secondary alcohols ($\text{CH}-(\text{OH})-$)
	4.72	75.4	C–H in methynes adjacent to ester groups ($\text{O}-\text{CH}=\text{}$)
	5.3	129.4	C–H in vinylic groups ($\text{CH}=\text{CH}$)
	6.45–7.30	111–122.8	aromatic signals

clearly visible. The latter are missing completely in the case of the hydrothermally pretreated bark.

The content of aliphatic hydroxyl groups of bark samples before and after enzymatic treatment was evaluated using quantitative ^{31}P NMR, by integrating the signals in the range of 149–146 ppm (Figure 4A).^{20,40} As the bark samples were partially soluble in NMR reagents, the obtained data that are described below refer only to the liquid phase and are used to compare the properties of the different samples. After enzymatic treatment, the aliphatic OH groups content increased and this change was more profound in case of hydrothermal pretreatment and steam exploded materials (from 0.8 mmol/g to 1.31 and 1.55 mmol/g, respectively) (Figure 4B), indicating the higher activity of esterases in these substrates. Study of the signals from acidic groups in the range of 135.5–134 ppm show an increase, although very low, in all samples, especially in steam exploded bark from 0.7 to 0.13 mmol/g (data not shown). This can be explained by the fact that labile glyceryl–ester bonds (hydroxycinnamic acid–glycerol- α,ω diacid) are more susceptible to enzymatic hydrolysis by cutinases than less reactive wax-type ester bonds between hydroxycinnamic acids and ω -hydroxy acids.¹⁸

Analysis of Suberin Samples. The $\{^{13}\text{C}-^1\text{H}\}$ HSQC spectrum of suberin isolated from bark samples with the main substructures depicted is presented in Figure 5A. Suberins isolated from bark subjected to different pretreatments showed identical signal pattern (Figure 5B), corroborating the idea that none of the pretreatment methods used had affected the nature of this polyester, at least in its oligomeric/monomeric form (after alkaline methanolysis). The obtained spectrum is characterized by the dominant presence of a major group of signals, in the range $\delta_{\text{C}}/\delta_{\text{H}}$ 24.2–33/1.22–2.43, associated with suberin methylenic groups, in different chemical environments, namely in the long aliphatic chains ($\delta_{\text{C}}/\delta_{\text{H}}$ 28.4–32.2/1.22–

Figure 6. (A) ^{31}P NMR spectrum of suberin isolated from *Betula pendula* steam exploded treated bark using 2-chloro-4,4,5,5-tetramethyl-1,3,2-dioxaphospholane as phosphorylating agent; IS, internal standard (cholesterol). (B) Aliphatic, condensed, guaiacyl, p -OH phenol and acidic groups content (mmol/g) as evaluated from ^{31}P NMR.

1.30), linked to hydroxylic and carboxylic moieties ($\delta_{\text{C}}/\delta_{\text{H}}$ 33.1/2.11–2.43 and 32.2–32.5/1.41–1.52) and nearby ester groups ($\delta_{\text{C}}/\delta_{\text{H}}$ 55.5/3.82) (Table 4). Other correlations observed were those assigned to C–H from methoxy groups ($\delta_{\text{C}}/\delta_{\text{H}}$ 51.2/3.61 and 63.4/3.98), aliphatic methyl groups ($\delta_{\text{C}}/\delta_{\text{H}}$ 15–15.5/0.60–0.95), allylic ($\delta_{\text{C}}/\delta_{\text{H}}$ 26.3/1.97) and vinylic groups ($\delta_{\text{C}}/\delta_{\text{H}}$ 129.4/5.3), and aromatic domains ($\delta_{\text{C}}/\delta_{\text{H}}$ 111–122.85/6.45–7.30). These data are in accordance with those being previously reported^{33,34} and are consistent with the structural features of the suberinic material.^{12,41,42}

^{31}P NMR analysis of suberin samples with 2-chloro-4,4,5,5-tetramethyl-1,3,2-dioxaphospholane allows quantification of aliphatic OH, condensed phenolic OH, guaiacyl phenol, p -hydroxyphenol and carboxylic acid content (Figure 6). All suberin samples were completely dissolved in both phosphorylating agents. Abundances were estimated by integrating the signals in the range of 149–146, 144.27–140.27, 140.24–138.8, 138.8–137.4 and 135.5–134 ppm, respectively, as previously described for suberin samples.⁴³ The ^{31}P NMR spectrum of isolated suberin from untreated bark contains high amounts of aliphatic OH groups, free functional groups of carboxylic acids, mainly attributed to suberin acidic monomers and polysaccharides that were coisolated with suberin, and guaiacyl structures, whereas p -hydroxyphenolic and condensed structures are detected in traces. After pretreatment, the amount of carboxylic OH groups decreases following polysaccharide removal, whereas signals from phenolic groups, attributed to

Figure 7. (A) ^{31}P NMR spectrum of suberin isolated from *Betula pendula* steam exploded treated bark using 2-chloro-1,3,2-dioxaphospholane as phosphitylating agent; IS, internal standard (*S*-norbornene-2,3-dicarboximide). (B) primary and secondary aliphatic OH group content in suberin samples as evaluated from ^{31}P NMR.

Figure 8. GPC chromatograms of suberin isolated from untreated and the solid fraction of pretreated *Betula pendula* outer bark samples.

the polyphenolic domain of suberin, increase. *p*-Hydroxyphenolic groups are completely absent, contrary to studies in potato tuber, where *p*-hydroxyphenyl and not syringyl structures were detected.⁴³ The change of primary and secondary aliphatic hydroxyl groups content before and after

Figure 9. Weight-average molecular weight (M_w), number-average molecular weight (M_n) and polydispersity index (PDI) of suberin samples.

pretreatment was evaluated by ^{31}P NMR from the samples phosphitylated with 2-chloro-1,3,2-dioxaphospholane, by integrating the signals in the range of 134.0–132.0 and 136.2–134.0 ppm, respectively.⁴⁴ The content of both decreased after pretreatment from 1.46 mmol/g to a range of 1.18–0.69 mmol/g (primary) and 2.93 mmol/g to range of 1.8–1.24 mmol/g (secondary) (Figure 7). The most profound decrease was observed in secondary structures, especially after steam explosion pretreatment, indicating the extensive cleavage of middle-chain methylenic bonds of suberinic fatty acids. Another possible reason leading to the decrease of OH groups in suberin from pretreated samples is the removal of small molecules that were released after pretreatment and washed out, leaving a solid fraction less rich in hydroxyls. This result is in accordance to the molecular weight distribution of the samples, as described below.

The molecular weight distribution of suberin samples is shown in Figure 8. Rather small polydispersities were obtained for suberin from untreated and hydrothermally pretreated bark, but many groups of higher MW polymers could be detected from the chromatograms of steam exploded and organosolv treated samples. The first strong peak in the untreated bark suberin chromatogram indicates the existence of a low molecular-weight fraction (nominally $M_n = 480$ Da) of low dispersity, with a small shoulder on 250 Da whereas the rest corresponds to a higher molecular weight and possessing a much wider distribution. Similar GPC profiles have been reported for suberin from *Quercus suber* cork and potato periderm.^{12,43} The GPC profile of hydrothermally pretreated bark is similar to that of the untreated material. The chromatograms of organosolv treated suberins are characterized by a strong peak of 250 Da and two large bands with a maximum at 1300 and 2700 Da. The GPC of suberin from steam exploded bark shows the existence of a fraction possessing a substantially higher molecular weight, revealing that other molecules, like lignin or smaller phenolic structures, may be isolated together with suberin. Chromatograms from the RID detector (data not shown) showed only one band at 45 kDa that is indicative of the presence of sugars linked to phenolic species; however, the band at 12.2 kDa is attributed to recalcitrant to alkaline methanolysis structural components that were coprecipitated with the suberinic fraction, as a result of condensation reactions that occur during steam explosion pretreatment.⁴⁵ The comparison of all suberins from pretreated samples, apart from HT suberin, shows that the untreated

Figure 10. Gas chromatogram of the methyl ester trimethylsilyl ether derivatives of suberin.

Table 5. Composition of the Low Molecular-Weight Volatile Fraction of Suberin from Untreated and Pretreated Outer Bark^a

	untreated	HT	SE	SE/OS 10:90	OS 50:50	OS 80:20
alkanedioic α,ω -acids	30.79	43.92	53.15	52.09	53.93	50.47
hexadecanedioic acid	0.41	n.d.	0.47	0.69	0.75	n.d.
octadec-9-enedioic acid	4.38	24.58	25.50	22.56	19.78	19.20
octadecanedioic acid	1.07	1.97	2.09	2.67	2.21	1.82
9,10-epoxy-octadecanedioic acid	14.99	2.50	9.70	8.87	14.02	9.16
9,10-dihydroxyoctadecanedioic acid	1.01	1.53	2.34	2.37	1.81	8.79
eicosanedioic acid	2.55	2.50	3.01	4.09	3.02	1.09
docosanedioic acid	6.38	10.84	10.04	10.84	12.34	10.41
hydroxyacids	64.66	52.45	45.52	47.5	43.39	46.24
10,16-dihydroxyhexadecanoic acid	2.82	1.59	0.70	1.56	1.66	n.d.
18-hydroxyoctadec-9-enoic acid	10.02	n.d.	1.79	1.74	n.d.	n.d.
9,10-epoxy-18-hydroxyoctadecanoic acid	2.63	1.50	1.39	1.99	1.55	2.36
9,10,18-trihydroxyoctadecanoic acid	28.27	35.03	17.31	18.79	19.78	23.45
20-hydroxyeicosanoic acid	4.16	2.40	4.24	5.48	4.68	3.24
22-hydroxydocosanoic acid	16.76	11.93	20.09	17.94	15.72	17.19
trans-ferulic acid	4.56	3.62	1.34	1.13	2.67	3.28
betulin	d.	d.	n.d.	d.	d.	d.
glycerol	d.	d.	d.	n.d.	n.d.	n.d.

^ad. detected in traces; n.d. not detected.

material contains a much higher proportion of the smaller fragments below 400 Da, corresponding to the monomeric units. Hence, the results suggest that under pretreatment conditions, suberin lower molecular weight fragments were solubilized and removed in the liquid fraction, leaving a solid fraction rich in oligomeric structures of higher molecular weight. Although the quantitative aspect related to these results is certainly incorrect because of the major difference in hydrodynamic volume between the structures of suberin and polystyrene that was used for calibration, the polymodal character of the GPC profile indicates the existence of major families of components in suberin. The weight-average molecular weight (M_w), number-average molecular weight (M_n) and polydispersity index (PDI) of suberin samples is shown in Figure 9.

Suberin monomers obtained after alkaline methanolysis were analyzed and characterized by gas chromatography coupled with mass spectrometric (GC–MS) analysis and were found to consist mainly of methyl ester/trimethylsilyl ether derivatives of 9,10-epoxy-octadecanedioic, octadec-9-enedioic acid, 9,10,18-

trihydroxyoctadecanoic acid, 22-hydroxydocosanoic acid and 18-hydroxyoctadec-9-enoic acid. A typical chromatogram of the suberin derivatives is shown in Figure 10. The identification and quantification of each component (relative with respect to the ionisability of each species under analysis conditions) is summarized in Table 5. Suberin composition is within the expected range for a *B. pendula* outer bark and *Q. suber* cork, with ω -hydroxyacids and α,ω -diacids as the main components and corresponding to 90–95% of the total amount.^{12,46–48} All these characterized components bear at least two OH and/or COOH groups, thus they represent interesting structures in terms of potential polycondensation monomers that can be used in further applications.⁸ Not the whole amount of suberin injected was detected as isolated components in the GC–MS chromatogram, as there were some fractions not ionizable enough at 85 eV and therefore went undetected. The same behavior was observed with suberin samples characterized before and reported in the literature.¹² Moreover, the difficulties in identification of all suberinic monomers can be partially attributed to the fact that methanolysis reaction leads

Figure 11. (A) ^{31}P NMR spectrum of lignin isolated from steam exploded *Betula pendula* outer bark using 2-chloro-4,4,5,5-tetramethyl-1,3,2-dioxaphospholane as phosphitylating agent. (B) Aliphatic, condensed, guaiacyl, *p*-OH phenol and acidic groups content (mmol/g) as evaluated from ^{31}P NMR. IS: internal standard (cholesterol).

Figure 12. GPC chromatograms of lignin isolated from untreated and the solid fraction of pretreated *Betula pendula* outer bark samples.

Figure 13. Weight-average molecular weight (M_w), number-average molecular weight (M_n) and polydispersity index (PDI) of lignin samples.

in the release of monomers and oligomers, but the GC–MS analysis only accounts for monomers and small oligomers (threshold of the used GC–MS system is 1500 Da) and therefore is only a partial characterization of the solubilized material. Small amounts of ferulate (4.56% in the untreated bark) were also found; trace signals from aromatic structures detected by $\{^{13}\text{C}-^1\text{H}\}$ HSQC analysis can be attributed to ferulic acid (FA) structures that are esterified to the aliphatic hydroxyacid chains of suberin. This value is substantially lower than the one determined by Py/TMAH analysis, which has been reported to reach 9% of the total suberin fraction.^{49,50}

Marques et al. (2015) have proposed that part of the FA is bound through ether bonds that are alkali resistant and not cleaved during methanolysis, and thus remains undetectable.³⁰ Glycerol was detected in traces though it is a major component of the suberin macromolecule;^{12,51,52} this is currently explained by the high water solubility of glycerol in light of the experimental procedure applied. After alkaline hydrolysis, the aliphatic acids were recovered in the organic phase whereas glycerol was discarded away in the aqueous phase. Analysis of methanolic extract prior to acidification showed in the past glycerol values up to 25% of the bark suberin mixture.⁵³

In the GC–MS chromatograms of suberin isolated from pretreated bark, the relative contents of 9,10-epoxy-18-hydroxyoctadecanoic, 9,10-epoxy-octadecanedioic and 10,16-dihydroxyhexadecanoic acid are lower when compared to hydroxyacids from untreated bark, whereas 18-hydroxyoctadec-9-enoic acid appears in traces. The total amount of alkanedioic α,ω -acids and hydroxyacids in suberin from untreated bark is 30.79% and 64.66% respectively, whereas after pretreatment hydroxyacid content reduces to 52.45–43.39%. This is attributed either to the fact that these compounds are cleaved and removed during the pretreatment, or they are subjected to condensation reactions and they are not detected as volatile compounds in the GC–MS analysis. Graça (2015), in an attempt to describe the primary structure of suberin, concluded that α,ω -diacids participate in glycerol- α,ω diacid–glycerol blocks that are the main backbones for the suberin structure, while ω -hydroxyacids are linked head-to tail and they are part of the end chain.⁵⁴ The latter makes ω -hydroxyacids susceptible to depolymerization.⁵⁵ Moreover, ω -hydroxyacids are considered to mediate between the aliphatic domain of suberin and the phenolic moieties through their esterification on ferulic acid molecules,^{19,56} so pretreatment, especially organosolv-type pretreatments, may cause degradation of these structures. Lower amounts of ferulic acid are also detected after pretreatment.

Table 6. Summary of the Methods Used and the Results Obtained in This Study

pretreatment	enzymatic treatment (cellulases, cutinases)	suberin chemical isolation	lignin chemical isolation
samples partially soluble in NMR workup reagents (gel-state analysis)	samples partially soluble in NMR workup reagents (gel-state analysis); solubility increased after enzymatic treatment	73–75% of extractive-free bark/samples soluble in NMR/GC–MS and partially soluble in GPC workup reagents	10–14% of extractive-free bark/samples soluble in NMR/GPC workup reagents
hydrothermal (HT) aliphatic structures remain, removal of polysaccharides ($\{^{13}\text{C}-^1\text{H}\}$ HSQC)	- removal of polysaccharides, partial cleavage of bonds in methoxy groups and β-O-4' structures ($\{^{13}\text{C}-^1\text{H}\}$ HSQC) - increase of aliphatic OH groups (^{31}P NMR) - removal of polysaccharides, partial cleavage of bonds in methoxy groups and β-O-4' structures, removal of methyl/methylene groups adjacent to polysaccharides ($\{^{13}\text{C}-^1\text{H}\}$ HSQC) - high increase of aliphatic OH groups (^{31}P NMR)	- 31% alkanedioic α,ω-acids and 65% hydroxyacids (GC–MS) - high proportion of the small monomeric units/fragments below 400 Da (GPC) - 44% alkanedioic α,ω-acids and 52% hydroxyacids (GC–MS) decrease of primary and secondary OH groups (^{31}P NMR) - high proportion of the small monomeric units/fragments below 400 Da (GPC)	- aliphatic OH groups and OH from guaiacyl structures mainly detected (^{31}P NMR) - higher molecular weight oligomeric fractions ($M_n = 2900$) (GPC) - higher content of acidic OH groups units and lower content of p-OH-phenols structures (^{31}P NMR) - polymodal M_n pattern, high proportion of the smaller fractions (GPC)
steam explosion (SE) aliphatic structures remain, removal of polysaccharides ($\{^{13}\text{C}-^1\text{H}\}$ HSQC)	- removal of polysaccharides, partial cleavage of bonds in methoxy groups and β-O-4' structures, removal of methyl/methylene groups adjacent to polysaccharides ($\{^{13}\text{C}-^1\text{H}\}$ HSQC) - high increase of aliphatic OH groups (^{31}P NMR)	- 53% alkanedioic α,ω-acids and 46% hydroxyacids (GC–MS) decrease of primary and secondary OH groups (^{31}P NMR) - polymodal M_n pattern, higher molecular weight oligomeric fractions detected (GPC)	- higher content of acidic OH groups units and lower content of p-OH-phenol structures (^{31}P NMR) - polymodal M_n pattern, higher molecular weight oligomeric fractions detected (GPC)
organosolv (OS) aliphatic structures remain (mainly CH_3), extensive cleavage of nonacylated β -O-4' structures compared to acylated, removal of polysaccharides ($\{^{13}\text{C}-^1\text{H}\}$ HSQC)	- removal of polysaccharides, complete cleavage of bonds in methoxy groups and β-O-4' structures, removal of methyl/methylene groups adjacent to polysaccharides ($\{^{13}\text{C}-^1\text{H}\}$ HSQC) - increase of aliphatic OH groups (^{31}P NMR)	- 50–54% alkanedioic α,ω-acids and 43–48% hydroxyacids (GC–MS) decrease of primary and secondary OH groups (^{31}P NMR) - polymodal M_n pattern, higher molecular weight oligomeric fractions together with low amounts of monomeric units detected (GPC)	- higher content of acidic OH groups units and lower content of guaiacyl structures (^{31}P NMR) - higher molecular weight oligomeric fractions ($M_n = 7300$) (GPC)

Analysis of Lignin Samples. Lignin isolated after dioxane/acidified water treatment was a brown material of low density obtained in a yield of 10–14% of the extractive-free bark. The functional groups of lignin isolated after acidolysis from the untreated and pretreated bark samples were evaluated by quantitative ^{31}P NMR. The lignin fraction from untreated bark shows high amounts of aliphatic OH groups, as well as OH groups from guaiacyl and *p*-hydroxyphenyl structures, whereas condensed phenolics are detected in traces. Lignin isolated from birch bark is mainly composed of guaiacyl-type alkylphenols; the same has been observed for lignin isolated from outer bark cork.⁵⁷ After pretreatment, the amount of carboxylic OH groups increases, whereas *p*-hydroxyphenyl structure content reduces. The data shown in Figure 11 indicate that all examined samples had a similar relative functional group distribution, with the exception of the untreated sample which showed lower content of acidic OH groups units and higher content of guaiacyl structures. When compared to the untreated sample, data from all treated samples showed a profound reduction in total phenol content, especially in *p*-hydroxyphenolic structures.

The molecular weight distribution of lignin samples shown in the GPC is illustrated in Figure 12A. The GPC profile of untreated bark lignin is dominated by a strong peak that indicates the existence of a high molecular-weight fraction (nominally $M_n = 2900$ Da) with small shoulders on 870, 380 and 250 Da. Lignin from all pretreated materials showed a polymodal pattern, with the exception of lignin from 80:20 EtOH/water organosolv fractionation that corresponds to highest weight-average molecular weight ($M_n = 7300$ Da) and possesses a much wider distribution. This can be attributed to the high proportion of organic solvent that was used during pretreatment (80% EtOH), that led to stronger chemical fragmentation on one hand, and to the solubilization and removal of lower molecular weight phenolic fragments in the liquid fraction on the other hand, leaving a solid fraction rich in structures of higher molecular weight.⁵⁸ The weight-average molecular weight (M_w), number-average molecular weight (M_n) and polydispersity index (PDI) of lignin samples is shown in Figure 12B. The ^{31}P NMR spectrum is displayed in Figure 13.

The results show that the isolated fraction is enriched in a lignin that still contains suberin and carbohydrates. The same has been observed for milled wood lignin isolated from cork.⁵⁸ It has been shown that, in most cases, polymeric carbohydrates cannot be completely removed by chemical treatments. The elimination of aliphatic suberinic acids of cork before lignin isolation would be a possible alternative to obtain pure lignin. 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.⁵⁹ It has also been reported that in the structure of birch bark exists a polymethylenic biopolymer with alkyl chain length consisting of long carbon chains, called suberan, which is nonhydrolyzable with different methods and thus can be coisolated with other cell wall components.⁶⁰

CONCLUSIONS

In this study, various analytical methods were used in order to reveal the structural changes in suberin and lignin fractions of *B. pendula* outer bark after applying different pretreatment methods. The methods used and the main results are summarized in Table 6. Ball-milling of the polymers was

crucial in order to obtain a homogeneous solution for structural characterization of the polymers. The $\{^{13}\text{C}-^1\text{H}\}$ HSQC spectrum of *B. pendula* bark showed that the main types of structural moieties include aliphatic chains from suberin aliphatic acids, carbohydrates and, to a lower extent, lignin. On the basis of data from ^{31}P NMR spectroscopy, it may be concluded that the aromatic moiety must contain a large amount of nonetherified G-type units that are esterified ferulates. An attempt to isolate chemically suberin and lignin from untreated and pretreated bark gave a “lignin-fraction” enriched in phenolic structures but still containing some associated aliphatic acids and carbohydrates and a “suberin fraction” consisted of monomeric and oligomeric structures with different molecular weight distributions. Evaluation of cutinase activity on birch outer bark revealed that the enzymes perform the cleavage of ester bonds resulting in reduction of methoxy and aliphatic groups in the remaining solid fraction, while the aromatic fraction remains intact.

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Notes

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ABBREVIATIONS

GC–MS = Gas chromatography–mass spectrometry
GPC = Gas permeation chromatography
HSQC = Heteronuclear single quantum coherence spectroscopy
HT = Hydrothermal pretreatment
NMR = Nuclear magnetic resonance
OS = Organosolv pretreatment
SE = Steam explosion

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