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Structural insights on laccase biografting of ferulic acid onto lignocellulosic fibers

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ABSTRACT

Treatment of high-kappa sisal pulp with *Trametes villosa* laccase and ferulic acid resulted in strong increases of kappa-number and acid-group content due to biografting of this phenolic acid, as shown by pyrolysis in the presence of tetramethylammonium hydroxide. The coupling linkages were investigated by 2D NMR of the lignin isolated from pulps. The aromatic region of the spectra showed incorporation of the cinnamic molecule, representing ~4% of the lignin content, that according to the displacement of its olefinic $^{13}C_{\beta}$–$^{1}H_{\beta}$ signal to 117.0/6.40 ppm would be C_4-etherified. The aliphatic region of the spectra showed that ferulic acid also incorporates as the corresponding β–β′ dilactone (another ~4% of the total lignin) with characteristic $^{13}C_{\alpha}$–$^{1}H_{\alpha}$ and $^{13}C_{\beta}$–$^{1}H_{\beta}$ correlations at 81.8/5.69 and 47.9/4.19 ppm, respectively. The sisal lignin in the treated pulps was only slightly modified (including a small increase of C_{α}-oxidized units) revealing that the main effect of the treatment was ferulic acid biografting.

1. Introduction

Laccases (benzenediol:oxygen oxidoreductase, EC 1.10.3.2) are multi-copper oxidases widely distributed in fungal and plant species, where they play multiple functions [1]. Laccases present a broad substrate specificity including substituted phenols, aromatic amines and thiols and many others, which are converted into reactive radicals using oxygen as the electron acceptor (and releasing water) [2]. By virtue of these characteristics, laccases are being intensively investigated as eco-friendly biocatalysts for a wide array of biotechnological applications [3]. Within the pulp and paper industry, laccases have arisen great interest, especially in combination with chemical mediators for delignifying (and bleaching) pulp [4–6]. Due to its molecular size and low redox potential, laccases can directly oxidize only the exposed phenolic moieties in the lignin polymer. The advantage of the use of mediators [7] is their ability to expand the activity toward the more recalcitrant non-phenolic lignin and overcome the accessibility restrictions of pulp cell walls. In addition to synthetic mediators, with some environmental risks due to potential toxicity, the so-called natural mediators have been largely investigated during recent years including phenolic compounds related to lignin [8].

A novel subject of research in the pulp and paper field is the application of laccase-catalyzed radical coupling reactions to modify lignocellulosic fiber chemistry with a view to altering paper properties [9]. Two main approaches are used for laccase-assisted modification of lignocellulosic fibers: (i) laccase-mediated cross-linking of lignin molecules in situ; and (ii) coupling of low molecular weight (generally phenolic) compounds onto fibers (*biografting*). The former approach has proven effective for the manufacture of binderless wood boards, where the bonding mechanism involves the enzymatic activation of lignin on fiber surfaces through the production of phenoxyl radicals which couple when the fibers are pressed into boards [10]. Other studies showed combination of laccase with lignin-rich extractives or different mediators to improve pulp wet strength, which was ascribed to both polymerization of added lignin and to production of phenoxyl radicals forming water-resistant linkages between fibers [11,12].

The second approach, which has been intensively investigated and coincides with that of the present study, provides a versatile method for functionalizing lignocellulosic fibers and imparting

Abbreviations: 2D NMR, two-dimensional nuclear magnetic resonance; ABTS, 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonate); DMSO, dimethylsulfoxide; FA, ferulic acid; G, guaiacyl; GC/MS, gas chromatography/mass spectrometry; HSQC, heteronuclear single-quantum correlation; S, syringyl; TMAH, tetramethylammonium hydroxide.

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desirable properties to pulps. Biografting of phenolic acids to kraft pulp fibers was found to increase dry strength properties in the resulting papers, which was ascribed to the ability of carboxyl groups to promote inter-fiber hydrogen bonding and fiber swelling [13]. Other studies have reported the development of antibacterial properties in different lignocellulosic substrates treated with laccase and simple phenols or tannins [14–16]. Laccase-catalysed grafting has also been applied to impart hydrophobicity to wood veneers and kraft pulp by using fluorophenols and lauryl gallate, respectively [17,18]. Although much research has been carried out to explore the potential of biografting for tailoring the properties of lignocellulosic materials, few of them have assessed the mechanistic aspects of this process and the nature of the chemical bonds formed [16,17] and they mainly involved the use of lignin model compounds due to the complexity of the lignin polymer.

In previous works [15,19,20] laccase biografting of simple phenolic compounds (such as syringaldehyde, acetosyringone and *p*-coumaric acid) on flax and sisal pulps was observed by the increase of both kappa number and Klason lignin content and, in the case of phenolic acids, by the increase of fiber anionic charge due to the presence of the carboxylic functionality. The covalent binding of these compounds on fiber components was confirmed by using an analytical approach based on pyrolysis of the whole treated fibers in the presence of tetramethylammonium hydroxide (TMAH), a method also known as thermochemolysis [21]. In the present study, a high-kappa pulp from sisal was treated with laccase and (*trans*) ferulic acid (FA) according to the conditions reported by Aracri et al. [20] as those providing the highest degree of grafting of this phenolic acid. After extensive washing, the treated pulp was directly analyzed by pyrolysis (in the absence/presence of TMAH) to confirm the FA incorporation. Then, in order to gain additional information on the amount of FA incorporated with respect to pulp lignin, and identify the lignin-FA linkages formed in the biografting reaction, analysis of the lignin isolated from treated pulp was performed by HSQC (heteronuclear single quantum correlation) 2D NMR spectroscopy. The results obtained are highly novel and relevant to the understanding of the biografting mechanisms during laccase-assisted modification of lignocellulosic materials.

2. Materials and methods

2.1. Enzyme and chemicals

Laccase from *Trametes villosa* was kindly provided by Novozymes (Bagsvaerd, Denmark) and frozen until use. One activity unit (U) was defined as the amount of enzyme transforming 1 μmol of 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonate) (ABTS) per minute to its cation radical ($\varepsilon_{436\,nm}$ = 29,300 M^{-1} cm^{-1}) in 0.1 M sodium acetate (pH 5) at 25 °C. Chemicals for enzyme assay were purchased from Sigma–Aldrich and used as received.

2.2. Pulp

Pulp was obtained from a laboratory cooking of sisal (*Agave sisalana*) fiber bundles kindly supplied by Celesa mill (Tortosa, Spain). The raw material was placed into a stainless steel rotating digester, where white liquor consisting of de-ionized water and NaOH was added to reach a liquid/solid ratio of 3.5. The digester was sealed, heated from room temperature to 160 °C in 60 min and kept isothermally for 45 min. Decreasing concentrations of NaOH were assayed (from 15% to 9% active alkali expressed as Na_2O) until obtaining a pulp with the highest possible lignin content. After cooling, the resulting products in the digester were filtrated with an 80 mesh screen for recovering the liquor and pulp. The pulp was thoroughly washed with tap water, defibrated in a defibrator chamber and then screened on a flat screen using a 0.2 mm slot screen.

Prior to initial characterization, pulps (2% consistency) were washed with diluted H_2SO_4 (pH 4) for 30 min, which was followed by filtration and extensive washing with de-ionized water. This step ensured removal of contaminants and metals, and brought the pulp to the pH required for the enzyme treatment.

Acid-washed pulps were disintegrated for 50,000 revolutions (ISO 5263), which was followed by filtration through a Buchner funnel, pre-refining for 5000 revolutions and refining for 4500 revolutions according to ISO 5264-2. The application of a pre-refining step, carried out using a 2 mm gap between the working surfaces of the PFI mill, was considered necessary to obtain a more homogeneous material and ensure an easier running of the PFI mill during refining.

2.3. Enzymatic treatments of pulps

Refined pulp samples were treated in an oxygen-pressurized (0.6 MPa) reactor at 5% consistency [20], using 50 mM sodium tartrate (pH 4), 40 U/g laccase and 3.5% (w/w) FA (all relative to pulp dry weight). Tween 80 (0.05%, w/v) was added as surfactant. Treatments were conducted for 4 h at 30 rev/min shaking, and 50 °C. Pulp samples treated under identical conditions in the absence of FA were used as controls. After treatment, the pulp samples were filtered in a fritted glass funnel and washed with de-ionized water until a colorless, neutral filtrate was obtained.

2.4. Analysis of pulp properties

Pulp properties were analyzed after Soxhlet extraction with acetone aimed at removing the fraction of FA that failed to covalently bind to fibers [19]. Kappa number and brightness were determined according to the standard methods ISO 302 and ISO 3688, respectively. The bulk acid group content was determined by conductimetric titration as described elsewhere [22]. In short, 1.5 g pulp was stirred in 300 ml of 0.1 M HCl for 1 h, followed by rinsing with deionized water in a finely fritted funnel. The sample was resuspended in 250 ml of 1 mM NaCl, spiked with 1.5 ml of 0.1 M HCl and titrated against 0.05 M NaOH in 0.25 ml increments, with conductivity measurement after each addition. All the reported results were the averages of two measurements.

2.5. Enzymatic isolation of residual lignin from pulps

Cellulolytic enzyme lignins were isolated by enzymatically saccharifying polysaccharides as described by Chang et al. [23]. Cellulysin (Calbiochem), a crude cellulase preparation from *Trichoderma viride* also containing hemicellulase activities, was used. Its activity was ≥10,000 units/g, estimated as reducing sugars (glucose equivalents) released from paper filter at 40 °C, pH 4. The extractives free ball-milled material (200 mg) was suspended in 20 mM NaOAc buffer (30 ml, pH 5.0) in a 50 ml centrifuge tube, 8 mg of Cellulysin was added, and the reaction slurry was incubated at 30 °C for 48 h. The solids were pelleted by centrifugation (8000 rpm, 4 °C, 20 min), and the process was repeated with fresh buffer and enzyme, two times. Finally, lignin was recovered by filtration, washed with ultrapure water and then lyophilized.

2.6. Analytical pyrolysis

Pyrolysis coupled to gas chromatography/mass spectrometry (GC/MS) of pulp samples was performed in the absence and the presence of TMAH. The pyrolysis was carried out using an EGA/PY-3030D micro-furnace pyrolyzer (Frontier Laboratories Ltd.) connected to an Agilent 7820A gas chromatograph using a