ENZYMATIC MODIFICATION OF LIGNIN AND LIGNIN FRACTIONS OBTAINED FROM PREATREATED BIOMASS

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Abstract

The potential for using *Trametes versicolor* laccase to graft the polysaccharide chitosan onto chemically fractionated lignin was examined to enhance the solubility of lignin and to obtain products with special properties. Due to the complexity of lignin molecule the reaction was first performed on lignin model compound with small molecular weight. The efficiency of the enzyme was investigated in the oxidative reactions of coniferyl alcohol with glucosamine.
Alcell Lignin was previously fractionated by acetone/water 50:50 (v/v) and the enzymatic modification of the soluble fraction with chitosan was carried out in the same solvent system. The modified lignin was isolated and the product was characterized by means of size exclusion chromatography and FT-IR in order to evidence the main lignin modifications after laccase coupling reactions. An important increase of the molecular weight and significant structural modifications were observed for the product obtained after the coupling reactions of Alcell Lignin with chitosane demonstrating the achievement of the modification reactions.

Introduction

Lignin, like cellulose and hemicelluloses, is a major component of plant materials and the most abundant form of aromatic carbon in the biosphere. From the chemical point of view, lignin is a heterogeneous, optically inactive polymer consisting of phenylpropanoid interunits, which are linked by several covalent bonds (e.g. aryl-ether, arylaryl, carbon-carbon bonds) [1]. Despite lignin's natural recalcitrance, a number of microorganisms are known to degrade lignin. [2]. Enzyme systems for the degradation of macromolecular lignin face several challenges. The substrate is a large heterogeneous polymer that necessitates attack by extracellular enzymes or agents. Lignin does not contain hydrolysable linkages, which means that the enzymes must be oxidative. Laccases (EC 1.10.3.2) are copper containing enzymes that can oxidize a variety of phenolic compounds including those typically found in lignin [3]. They provide an attractive means to modify the physical and chemical properties of lignin, e.g. by altering solubility, surface properties, and hydrophobicity of the polymer via oxidation. Potential options to increase the value of isolated lignin include addition of key functionalities or molecules directly to the lignin, thereby increasing its versatility. The activity of laccases on small lignin model compounds has been reported in several publications [4]. It has been proved that lignin fractions can also have a number of useful properties, like antioxidant character, and by grafting of various molecules can induce new functions and properties. The aim of our studies was to obtain modified lignin fractions with potential utilization as biomaterials

Experimental

Chemicals, enzyme and lignin.

Glucosamine hydrochloride (98%), laccase from *Trametes versicolor* (30.6 U mg^{-1} of solid), lignin peroxidase (LiP, 0.18 U mg^{-1} of solid), coniferyl alcohol (98%), veratryl alcohol, chitosan (Flonac C, M=100000) and the substrate syringaldazine were purchased from Sigma-Aldrich (Taufkirchen, Germany). Organosolv lignin from mixed hardwoods (Alcell) was obtained from Repap Technologies Inc. (Valley Forge, PA, USA). Horseradish peroxidase II (HRP, 181 Purpurogallin units/mg) was purchased from Sigma Chemical St. Louis, MO.

Enzyme assay

Lacase activity was determined spectrophotometrically using syringaldazine as substrate in different acetone-water mixtures. The reaction mixture (1 ml) contained 0.027 mM syringaldazine dissolved in acetone-water 50 %(v/v) and 0.5 μg ml^{-1} laccase. The formation of the syringaldazine radical (ε_{530}= 65 $mM^{-1}cm^{-1}$) was followed in time at 530 nm and 25°C [5].
Lignin peroxidase (LiP) and *Horseradish peroxidase (HRP)* activities were assayed spectrophotometrically using veratryl alcohol (for LiP) and coniferyl alcohol (for HRP) as substrates in 0.1M sodium citrate-phosphate pH=3 (for veratryl alcohol) and 0.1M sodium acetate trihydrate pH=5 (for coniferyl alcohol), respectively. The reaction mixture (1 ml) contained veratryl alcohol/coniferyl alcohol dissolved in buffer and 10 μg LiP/ 0.25 μg HRP solubilized in buffer. The oxidation of the veratryl alcohol was followed in time at 310 nm using ε_{310}= 9.3 $mM^{-1}cm^{-1}$, respectively at 262 nm for coniferyl alcohol consumption using ε_{262}= 14000 $M^{-1}cm^{-1}$. The increase in absorbance is followed at 310 nm and 25°C, to determine the LiP activity in international units meanwhile for coniferyl alcohol the decrease in absorbance was followed at 262 nm and 25°C, to determine the HRP activity.

Enzymatic modification of coniferyl alcohol with glucosamine using laccase as catalyst.

The covalent coupling of coniferyl alcohol with glucosamine was studied using laccase as catalyst in 50:50 (v/v) acetone/air-saturated water. The typical experimental conditions were as follows: 100 mg coniferyl alcohol; 200 mg glucosamine hydrochloride (molar ratio coniferyl alcohol to co-substrate1:2), 0.24 mg ml^{-1} laccase dissolved in air-saturated water, reaction volume 10 ml. The reaction was started by the addition of laccase solution and the reaction mixture was stirred at 20 °C for 24 hours. Two control reactions without enzyme and without the co-substrate, respectively, were carried out at the same conditions. The coniferyl alcohol consumption was monitorised by HPLC analysis.

Enzymatic modification of Alcell lignin using laccase.

The covalent coupling of a low molecular weight Alcell lignin sample with chitosane was studied using laccase as catalyst in 50:50 (v/v) acetone/air-saturated water. The isolated lignin fraction soluble in acetone/water 50:50 (v/v) were used as the starting substrates for laccase modification reactions.The typical experimental conditions were as follows: 100 mg Alcell lignin fraction, 200 mg chitosan Flonac C, 0.24 mg ml^{-1} laccase dissolved in air-saturated water, reaction volume 10 ml. Since the chitosan is not soluble in acetone-water mixture it was suspended in the reaction mixture and stirred at room temperature for 24 hours. The solid was filtrated and washed with acetone for several times to remove the adsorbed lignin, dried and analyzed. Two control reactions were carried out at the same conditions. Reactions were stopped by adding 50 ml deionized water and lignin precipitation by lowering the pH to 1.0 with 1M HCl. The reaction products were separated on a glass filter (G4), washed two times with acetone to remove the soluble unreacted substrates and dried overnight in a vacuum oven at 60°C. All experiments were carried out at least in duplicate.

Fourier transform infrared spectroscopy (FT-IR).

Fourier Transform Infrared (FT-IR) spectra of the lignin samples were obtained in attenuated total reflectance (ATR) mode on a Varian Scimitar 1000 FT-IR spectrometer equipped with a DTSG-detector PIKE MIRacle ATR equipped with a diamond w/ZnSe lens single reflection plate. Spectra were collected in the range 4000-650 cm^{-1} with a resolution of 4 cm^{-1} and with 128 co-added scans. The spectra were baseline corrected and normalized to 1510 cm^{-1}. Shoulders and complex bands were deconvoluted for a good assessment. The assignment of peaks was performed as described by Faix (1991) and Boeriu et al. (2004) [6,7].

Size exclusion chromatography (SEC).

The molar mass distribution of lignins was analyzed by alkaline SEC using a TSK gel Toyopearl HW-55F column, 0.5 M NaOH as eluent, UV detection at 280 nm and calibration with sodium-polystyrene sulfonates, according to the procedure described elsewhere [8]. Mw (weight-average molecular weight), Mn (number average molecular weight) and polydispersity (PD, Mw/Mn) were calculated.

High Performance Liquid Chromatography (HPLC)

The HPLC analysis was performed using a HPLC equipped wih UV detector (Waters 2487 Dual l absorbance detector) operating at 260 nm. A reversed phase column was used (Inersil ODS-2 5mm, 4.6 × 250 mm GL Sciences Inc. Tokyo, Japan). Mobile phase was water and acetonitrile suplemented with phosphoric acid (0.01 % v/v) at the ratio of 80:20 (isocratic). Analysis was performed at 30^{o}C and mobile phase flow rate of 0.8 mL/min. Retention time of coniferyl alcohol was 12.1 min.

Results and discussion

1. Selection of biocatalyst

Laccase and peroxidase oxidize phenolic substrates, including lignin, to phenoxy radicals with concomitant reduction of oxygen or hydrogen peroxide, respectively. The phenoxy radicals generated in situ are unstable, and reactions involving polymerization as well as depolymerization of lignin can take place. Many phenols are enzymatically oxidized into homopolymers, but in the presence of lignin coupling between lignin and the phenols occurs. The influence of acetone on the laccase, lignin peroxidase and horseradish peroxidase catalytic activity was studied.

1.1. Kinetic study of syringaldazine enzymatic oxidation

Enzymes speed up the rate of a reaction by a definite amount, proportional to quantity of enzyme present. To measure reaction rate, some property difference between reactant and product must be identified. Rate can be measured as disappearance of reactant or accumulation of product [9]. To better understand the organic solvent effect on the kinetic mechanism in the laccase oxidation reaction several experiments were conducted with different syringaldazine concentrations. In previous study we have shown that laccase from Trametes versicolor showed good stability in high acetone concentrations (50% vol) and the oxidative polymerization reactions were efficient using the acetone: water 50: 50 (v/v) soluble lignin fractions with low molecular weights (Mw < 4000 g/mol) and dispersities [10]. The kinetic studies were conducted in 50:50 (v/v) acetone/air-saturated water, at 25 ^{o}C and different syringaldazine concentrations (10 - 90 μM) the enzyme concentration being maintained constant (0.5 μg mL^{-1}).

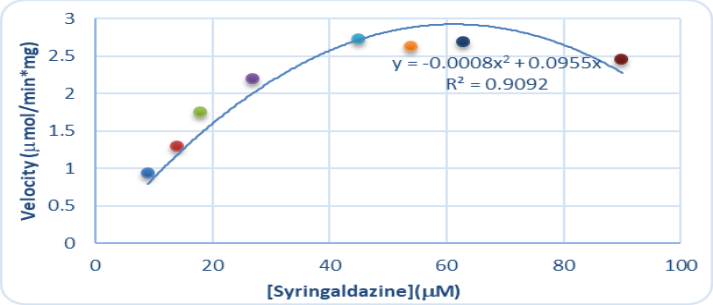

Figure 2. Michaelis-Menten kinetic curve in the oxidation reaction of syringaldazine using laccase as catalyst

From Figure 2 could be observed a hyperbolic behaviour of the substrate saturation curve, confirming the enzyme follows Michaelis-Menten behaviour. The Km value can be understood as enzyme's affinity for the substrate and was found to be 29,1 µM meanwhile the velocity V_{max} is 4,1 µmol min $^{-1}$ mg^{-1}.

1.2. The influence of the reaction media on the catalytic efficiency of lignin peroxidase and horseradish peroxidase.

The catalytic activity of lignin peroxidase and horseradish peroxidase was assayed spectrophotometrically using coniferyl alcohol and veratryl alcohol as substrate. The HRP and LiP activity decreased dramatically when acetone was introduced in the reaction media. Because of enzyme inhibition by the reaction solvent HRP and LiP could not be used in the coupling reaction of lignin with different compounds.

2. Covalent coupling of coniferyl alcohol with glucosamine

Due to the complexity of lignin macromolecule first we attempted to enzymatically modify model compounds with lower molecular weight. Laccase modification of coniferyl alcohol with glucosamine in 50 % vol. acetone-water mixture was monitored by HPLC analysis. Coniferyl alcohol consumption during the enzymatic reaction with glucosamine was compared with two control reactions without enzyme and without the co-substrate, respectively (Figure 3).

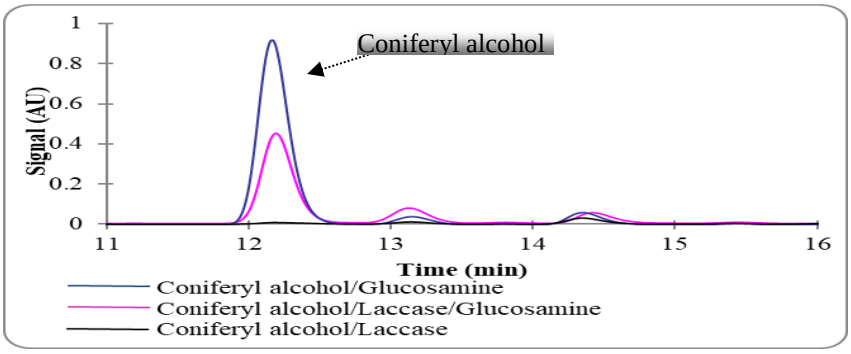

Figure 3. The HPLC chromatogram of the coniferyl alcohol modification reactions with glucosamine using laccase as catalyst

By comparing the obtained chromatograms, it can be observed that reactions take place different. In the reaction control without laccase were not observed any changes of the coniferyl alcohol meanwhile in the reaction of coniferyl alcohol with enzyme the substrate was entirely consumed (Figure 3- black line). In the case of the glucosamine coupling reaction, the reaction is different, only a certain amount of alcohol has been transformed

demonstrating that the coupling reaction take place. The reaction product could not be separated by means of HPLC analysis due to the substrate polymerization the resulting products having high molecular weights.

Covalent coupling of Alcell lignin with chitosan

The production lignin-chitosan conjugates using laccase as catalyst is challenging having a great impact on chitosan applications. The lignin-chitosan conjugates can improve the properties of chitosan such as enhanced antimicrobial properties with use in antimicrobial hydrogels or in packaging as well they can improve the optical response having a potential use as pigmenting materials and fillers in paints and coatings. Since the chitosan is not soluble in acetone-water mixture having great solubility in acid media, the chitosan was suspended in the reaction mixture and stirred at room temperature for 24 hours. The solid was filtrated and washed with acetone for several times to remove the adsorbed lignin, dried and analysed. Both the supernatant and the obtained solid phase were analysed by means of FT-IR (Figure 4) and SEC (Table 1) analysis.

Table 1. Molecular weight distribution of Alcell lignin before and after enzymatic coupling reactions with chitosan

	Mw	Mn	Mw/Mn
Alcell-50	2346	264	8.9
Alcell-50/Chitosan	2330	282	8.3
Alcell-50/Laccase	5708	714	8.0
Alcell-50/Laccase/Chitosan	10091	858	11.8

During the reaction coupling of Alcell lignin with chitosan the suspended chitosan changed from white in slightly brown. The SEC analysis of liquid phase (supernatant) revealed a significant increase of the molecular weight and polydispersity subsequent to the reaction as compared with the control reactions, that could be concluded by the coupling of chitosan residues to the lignin backbone. Unfortunately, we encountered solubilization problems and thus the characterization of the obtained product became difficile. FT-IR spectroscopy provided evidence that chitosan was grafted onto oxidized lignin by the apparition of a new absorption band at 1122 cm^{-1} and an increase in the intensity of the absorption band from 1500 cm^{-1} as compared with the control product and starting material. Follow up studies will be performed to optimize the reaction conditions and to isolate and characterize the formed products.

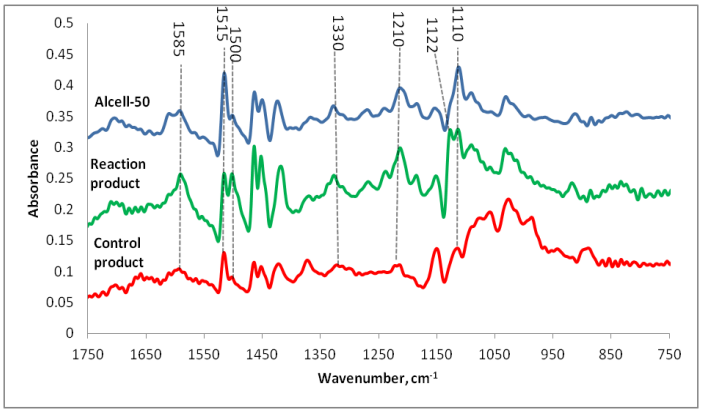

Figure 4. Comparison between the FT-IR deconvoluted spectra of the reaction product obtained in the enzymatic coupling of Alcell lignin with chitosan, the starting material and reaction control without laccase

Conclusion

Utilization of 50% concentration of acetone in the reaction mixture doesn't affect the activity of laccase in modification reactions. This finding opens up further possibilities for the utilization of laccases in areas where the solubility of the reactants or products is limited. Enzymatic modification of Alcell lignin with chitosan using laccase as catalyst looks promising. The exact mechanisms and chemical factors that contribute to the enzymatic modification of lignin with chitosan still require additional research. This study demonstrated the occurrence of structural changes of the lignin generated by the treatment with chitosan in a medium containing an organic co-solvent, employing laccase as catalyst.

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