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Inhibitory effect of lignin during cellulose bioconversion: The effect of lignin chemistry on non-productive enzyme adsorption

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HIGHLIGHTS

- Lignin was isolated from pretreated and native spruce and wheat straw.
- Homogeneous, ultrathin lignin films were successfully generated by spin-coating.
- Monocomponent cellulase adsorption on the lignin films was studied with QCM.
- Highest cellulase adsorption was detected on pretreated lignin films.
- Lower lignin-binding was detected when using enzyme without CBM and linker domain.

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ABSTRACT

The effect of lignin as an inhibitory biopolymer for the enzymatic hydrolysis of lignocellulosic biomass was studied; specially addressing the role of lignin in non-productive enzyme adsorption. Botanical origin and biomass pre-treatment give rise to differences in lignin structure and the effect of these differences on enzyme binding and inhibition were elucidated. Lignin was isolated from steam explosion (SE) pre-treated and non-treated spruce and wheat straw and used for the preparation of ultrathin films for enzyme binding studies. Binding of *Trichoderma reesei* Cel7A (CBHI) and the corresponding Cel7A-core, lacking the linker and the cellulose-binding domain, to the lignin films was monitored using a quartz crystal microbalance (QCM). SE pre-treatment altered the lignin structure, leading to increased enzyme adsorption. Thus, the positive effect of SE pre-treatment, opening the cell wall matrix to make polysaccharides more accessible, may be compromised by the structural changes of lignin that increase non-productive enzyme adsorption.

1. Introduction

Lignocellulose is the most abundant type of biomass in the biosphere and its utilisation for chemical and fuel production has gained increasing interest worldwide. The current liquid fuel, chemical, material and energy production primarily relies on non-renewable resources, which are unsustainable from both societal and environmental point of view. There is thus an urgent need to develop environmentally sound processing technologies enabling the production of fuels and wide variety of chemicals from lignocellulosic biomass. The biochemical processing of lignocellulosic biomass aims at enzymatic depolymerisation of cellulose and hemicellulose down to mono- and oligomeric sugars that may be further converted into various desired chemical products.

Lignin is an aromatic cell wall polymer in vascular plants. It encrusts and glues together the network of cell wall carbohydrates thus stiffening the cell wall structure. Its biosynthesis occurs by radical coupling of the lignin precursors: coniferyl, sinapyl and *p*-coumaryl alcohol that give rise to a random sequence of guaiacyl (G), syringyl (S) and hydroxyphenyl (H) subunits in the polymer,

Abbreviations: EnzHR, enzymatic hydrolysis residue; QCM, quartz crystal microbalance; SE, steam explosion; CBM, carbohydrate binding module; EMAL, enzymatic mild acidolysis lignin; WCA, water contact angle.

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respectively. The ratio of G-, S- and H-units differs in softwood, hardwood and grass species: softwood lignin is mainly composed of G-units whereas both G- and S-units are abundant in hardwood. In addition to G- and S-units, H-units are also present in grasses and compression wood lignin. Lignin is covalently linked to heteropolysaccharides both in trees and in herbaceous plants.

Biomass pre-treatment is a prerequisite for an efficient enzymatic hydrolysis of lignocellulose. Steam explosion (SE) pre-treatment is a widely exploited process that renders the cell wall carbohydrates accessible for hydrolytic conversion mostly by disrupting the heteropolysaccharide–lignin network surrounding the cellulose fibrils (Donaldson et al., 1988). SE pre-treatment is an extensively studied process in which the lignocellulosic biomass is heated by high-pressure saturated steam followed by a quick "explosive" decompression. Biomass impregnation with an acid catalyst, e.g. SO_2 prior to the SE is known to further improve enzymatic digestibility of the raw materials, particularly those of softwood origin (Clark and Mackie, 1987). Steam explosion partially solubilises heteropolysaccharides, but preserves most of cellulose and lignin in solid state. Studies have shown SE pre-treatment to delocalise lignin into aggregated droplets on the surfaces of plant cells (Donaldson et al., 1988) and radically change the lignin chemistry by cleaving β-O-4 aryl ether bonds and forming new C–C bonds (Robert et al., 1988).

In the enzymatic hydrolysis of pretreated materials, cellulases tend to bind on the lignin-rich surfaces (Palonen et al., 2004; Yang and Wyman, 2006), which is considered to be not only inhibitory for the degradation process, but also harmful for cellulase recycling. Enzyme-lignin interactions have been studied widely (for review, see Nakagame et al., 2011c), however, the fundamental reasons to explain the negative effect of lignin on the enzymatic hydrolysis are yet to be elucidated. Despite the wide use of SE pre-treatment, to our knowledge, only one publication is available concerning the effect of pre-treatment severity on lignin and its inhibitory properties (Nakagame et al., 2011b). Nakagame et al. (2010) showed that lignins isolated from woody species resulted in more inhibition in Avicel hydrolysis compared to lignin isolated from an herbaceous plant (corn stover) suggesting that native differences in lignin structure may give rise to differences in their inhibitory properties. Proper understanding of the lignin structures promoting enzyme adsorption could help to design better pre-treatment strategies that would alleviate the negative effects of lignin.

The aim of this study was to elucidate the effect of lignin chemistry on cellulase adsorption. *Trichoderma reesei* cellulase, cellobiohydrolase 1 (Cel7A) and its core domain were considered with regards to their interactions with lignins isolated from wheat straw and spruce before and after SE pre-treatment.

2. Methods

2.1. Biomass pre-treatment

Wheat harvest residue obtained from MTT Agrifood Research (Jokioinen, Finland) and spruce chips (*Picea abies*) obtained from UPM Research Centre (Lappeenranta, Finland) were air-dried and milled through a 1-cm screen with a cutting mill (Kamas Industri Ab, Sweden). The milled materials were pre-treated using steam explosion in a 10 L pressure tank (Haato Oy, Finland) equipped with a 5 L wire mesh basket to hold the biomass sample. Steam was fed in the tank until 15.5 bar (200 °C) pressure was achieved followed by fast release of pressure after a 10 min residence time. Solids were collected from the basket, air dried and milled through a one mm screen with a Wiley mill (Arthur H. Thomas Co., PA, USA).

2.2. Lignin isolation: enzymatic mild acidolysis lignin (EMAL) and lignin-rich hydrolysis residues (EnzHR)

The enzymatic mild acidolysis lignin (EMAL) isolation procedure was modified from Guerra et al. (2006) and Wu and Argyropoulos (2003). The milled materials were extracted with acetone (17 siphon cycles) using a large-scale Soxhlet system followed by air-drying and ball-milling. Milling was carried out using a rotary ball-mill KM1 (Scanteknik Oy, Finland) equipped with an 11 L rubber-coated jar. Air-dry biomass (200–250 g) was milled with industrial-grade Ø10 mm ceramic balls using a ball to sample weight ratio of 16.6, 60 rpm speed and a 14 days milling time. The original EMAL lignin isolation protocol involves enzymatic hydrolysis of the ball-milled materials with a fixed enzyme dosage (40 FPU/g d.m.) (Guerra et al., 2006). In this work the optimal enzyme dosage for each material was first screened in small-scale (3 mL) hydrolysis experiments (in 3% w/v dry matter, at 45 °C in 50 mM sodium acetate pH 5 and magnetic stirring) using a mixture of commercial enzyme preparations: Celluclast (1–10 FPU/g d.m.) and Novozym 188 (Novozymes Inc., Denmark). Constant dosage of β-glucosidase activity (450 nkat/g dry weight, corresponding to 7.9 mg/g in protein) from a Novozym 188 preparation was employed and the solubilised monosaccharides were assayed using a colorimetric assay of reducing sugars (Lever, 1972). β-Glucosidase activity was assayed from the Novozym 188 preparation as described in Bailey and Linko (1990). The hydrolysis degree was calculated by comparing the amount of liberated monosaccharides to the theoretical maximum yield. The theoretical maximum yield was determined for each material by summing the enzymatically hydrolysed carbohydrates with the carbohydrates remaining in the enzymatic hydrolysis residue. The carbohydrates in the residue were analysed as described in the Section 2.4. The large-scale enzymatic hydrolyses for EMAL lignin isolation were carried out in several batches in a shaking incubator (150 rpm, at 45 °C) using a 300 mL total volume and 6% (w/v) dry matter content. After hydrolysis the remaining lignin-rich residue (crude lignin) was separated, washed two times with water (pH adjusted to 2 with 1 M HCl) and freeze–dried.

The EMAL lignins were extracted from the crude lignin with 85:15 dioxane:water acidified with 0.01 M HCl. Part of the crude lignin was protease-treated according to Rahikainen et al. (2011) and freeze–dried to yield lignin-rich enzymatic hydrolysis residues (EnzHR).

2.3. Enzyme preparations

T. reesei Cel7A (CBHI) and the corresponding core enzyme were purified from the culture supernatant of *T. reesei egl1*$^-$, *egl2*$^-$ deletion strain kindly provided by Roal Oy (Rajamäki, Finland). *Tr*Cel7A was purified using a bentonite clay treatment at pH 4 followed by buffer exchange to 10 mM sodium–phosphate pH 7.2 with a Sephadex G-25 coarse gel filtration column (GE Healthcare, UK). Final purification step included an anion exchange step with a DEAE Sepharose FF column (GE Healthcare, UK) in 10 mM sodium-phosphate buffer pH 7.2. Following salt gradients (NaCl) were used in the run: 0–50 mM, 5 column volumes (CV); 50 mM, 2 CV; 100 mM, 2 CV; 100–250 mM, 5 CV; 250 mM 5 CV. Fractions containing highly pure *Tr*Cel7A were eluted during the 100–250 mM salt gradient. *Tr*Cel7A-core enzyme was purified according to Suurnäkki et al. (2000). *Thermoascus aurantiacus* Cel3A β-glucosidase was kindly provided by Roal Oy (Rajamäki, Finland). The *Ta*-Cel3A was produced in a *T. reesei cbh1*$^-$, *cbh2*$^-$, *egl1*$^-$, *egl2*$^-$ deletion strain and enzyme purification was carried out by heat treatment (60 °C, 2 h, pH 6).