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Lignin-derived inhibition of monocomponent cellulases and a xylanase in the hydrolysis of lignocellulosics

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HIGHLIGHTS

- The binding mechanisms of enzymes onto lignin thin films are enzyme specific.
- Of the studied enzymes, the hydrolysis yields of *Tr*Cel6A and *Tr*Cel7B were most affected by lignin.
- Soluble compounds from isolated lignin increased β-glucosidase activity up to 28%.

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ABSTRACT

Non-productive enzyme binding onto lignin is the major inhibitory mechanism, which reduces hydrolysis rates and yields and prevents efficient enzyme recycling in the hydrolysis of lignocellulosics. The detailed mechanisms of binding are still poorly understood. Enzyme-lignin interactions were investigated by comparing the structural properties and binding behaviour of fungal monocomponent enzymes, cellobiohydrolases *Tr*Cel7A and *Tr*Cel6A, endoglucanases *Tr*Cel7B and *Tr*Cel5A, a xylanase *Tr*Xyn11 and a β-glucosidase *An*Cel3A, onto lignins isolated from steam pretreated spruce and wheat straw. The enzymes exhibited decreasing affinity onto lignin model films in the following order: *Tr*Cel7B > *Tr*Cel6A > *Tr*Cel5A > *An*Cel3A > *Tr*Cel7A > *Tr*Xyn11. As analysed in Avicel hydrolysis, *Tr*Cel6A and *Tr*Cel7B were most inhibited by lignin isolated from pretreated spruce. This could be partially explained by adsorption of the enzyme onto the lignin surface. Enzyme properties, such as enzyme surface charge, thermal stability or surface hydrophobicity could not alone explain the adsorption behaviour.

1. Introduction

Environmental and societal concerns have increased an interest to use renewable resources for production of fuels and chemicals. For converting lignocellulosic biomass into sugars, enzymatic hydrolysis is presently considered as the most promising technology. Steam pretreatment is the most commonly used pretreatment method in the present demo and commercial scale biorefineries. During steam pretreatment lignin is partly solubilised, however, the major part of lignin is not removed, but redistributed and chemically modified (Donaldson et al., 1988). Although steam pretreatment is essential to enhance polysaccharide accessibility to the hydrolytic enzymes, lignin in pretreated biomass impairs hydrolysis by physically blocking the enzyme access to polysaccharides and non-productively binding enzymes. In addition, soluble phenolic compounds released in the pretreatment may affect the activity of enzymes.

Lignin is formed via radical coupling of three monolignols (p-coumaryl alcohol, coniferyl alcohol and sinapyl alcohol), which give rise to the aromatic units, guaiacyl (G), syringyl (S) and hydroxyphenyl (H), in the lignin polymer. Softwood lignin contains mainly G units whereas annual plants contain all three units in varying composition. Steam pretreatment decreases the β-O-4 linkages in lignin and also facilitates new bond formation through condensation reactions increasing the overall molecular size of lignin (Li et al., 2007). Especially lignin condensation, which is more pronounced with softwood-type lignin, has been shown to decrease hydrolysis yields (Pielhop et al., 2015). The negative effect of lignin can been reduced by introducing chemical additives to steam pretreatment that modify lignin (Chandra et al., 2015; Pielhop et al., 2015) or by engineering the lignin synthesis pathway to increase the amount of S-type lignin (Li et al., 2010).

Enzyme-lignin interaction have been explained mainly by hydrophobic (Eriksson et al., 2002; Palonen et al., 2004) and

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electrostatic interactions (Berlin et al., 2006; Nakagame et al., 2011a). Many fungal cellulases have a two domain structure containing a catalytic domain and a conserved carbohydrate binding module (CBM) from the CBM family 1 (van Tilbeurgh et al., 1986). The two domains are connected by a highly glycosylated linker. The catalytic domain and CBM are both involved in enzyme binding onto lignin, although the CBMs have a more profound role in the binding (Palonen et al., 2004; Rahikainen et al., 2013b). In family 1 CBMs, the planar cellulose binding surface is highly conserved containing aromatic and charged amino acid residues involved in binding onto cellulose (Hoffrén et al., 1995). The planar surface of the CBM of *Tr*Cel7A contains three tyrosyl residues (Y5, Y31 and Y32), which are essential in binding of the CBM onto cellulose with some contribution from charged residues (Linder et al., 1995). The same aromatic residues have also been shown to be involved in enzyme binding onto lignin (Gao et al., 2014; Rahikainen et al., 2013a). Recently, Sammond et al. (2014) have reported that, the size and amount of hydrophobic areas on an enzyme core and CBM correlate positively on binding onto lignin.

Less data is available on the role of electrostatic interactions on the enzyme binding onto lignin. Nakagame et al. (2011b) observed that positively charged cellulases adsorbed more onto the negatively charged lignin at pH 4.8. The charge of lignin is mainly affected by the deprotonation of phenolic hydroxyl and carboxylic acids groups in lignin. In hydrolysis conditions, around pH 5, lignin is mainly negatively charged and elevating the pH increases the negative charge of lignin. Cellulases with low pI or pretreatments that introduce negatively charged groups to lignin, have been suggested to reduce cellulase adsorption onto lignin (Nakagame et al., 2011a). Yamaguchi et al. (2016) identified short peptide sequences prone to adsorption onto isolated lignin. Especially the presence of prolines and positively charged histidines in the peptide sequences increased the affinity. In addition, elevating the temperature increases the adsorption of enzymes onto lignin by both enhancing the adsorption and strengthening the enzyme-lignin interactions (Rahikainen et al., 2011). According to the studies of Rahikainen et al. (2013c), thermostable enzymes are more tolerant for lignin inhibition. Surfactants (Eriksson et al., 2002) and additional proteins like BSA (Yang and Wyman, 2006) have been shown to prevent non-productive enzyme binding onto lignin.

This work aims at understanding the interactions between model lignins and structurally different enzymes needed in the hydrolysis of lignocellulose. Industrially relevant cellulases, cellobiohydrolases (CBHs) *Tr*Cel7A (CBH I), *Tr*Cel6A (CBH II), endoglucanases (EGs) *Tr*Cel7B (EG I), *Tr*Cel5A (EG II), a *Trichoderma reesei* xylanase *Tr*Xyn11 (XYN II) and an *Aspergillus niger* β-glucosidase *An*Cel3A (BGL), were selected for this work. Well-characterised lignins isolated from steam pretreated spruce (SPS) and wheat straw (SPWS) were used in binding and hydrolysis experiments.

2. Materials and methods

2.1. Lignocellulosic materials and lignin isolation

Enzymatic mild acidolysis lignin (EMAL) was isolated from steam pretreated spruce (SPS) and wheat straw (SPWS) according to Guerra et al. (2006) and Wu and Argyropoulos (2003) with the modifications described by Rahikainen et al. (2013b). Shortly, steam pretreatment for spruce and wheat straw was performed at 200 °C for 10 min without an acid catalyst. The ball milled extractive-free lignocellulose was enzymatically hydrolysed and lignin was extracted with mildly acidic dioxane-water from the hydrolysis residue. For EnzHR lignins, the starting materials, spruce and wheat straw, were pretreated in industrially relevant conditions and the lignins were isolated with an extensive enzymatic hydrolysis followed by a protease treatment to remove solid-bound enzymes as described in (Rahikainen et al., 2013a).

2.2. Enzyme purification

The cellobiohydrolases (CBHs), endoglucanases (EGs) and xylanase were produced in-house by the fungus *T. reesei*. The CBHs *Tr*Cel7A (CBH I), *Tr*Cel6A (CBH II), EGs *Tr*Cel7B (EG I) and *Tr*Cel5A (EG II) were produced and purified as described in Suurnäkki et al. (2000) and the xylanase *Tr*Xyn11 (XYN II) according to Tenkanen et al. (1992). *Aspergillus niger* β-glucosidase *An*Cel3A (BGL) was purified from a commercial product Novozym 188 (Novozymes, Bagvaerd, Denmark) according to Sipos et al. (2010). A total of 36.6 mg of protein was recovered with 39% of original β-glucosidase activity remaining. The identity of the β-glucosidase was verified using peptide mass fingerprinting. The protein was digested using trypsin and the recovered peptides were analysed with Autoflex II LRF50-CID (Bruker, CA, USA) matrix-assisted laser desorption/ionization with time of flight mass spectrometry (MALDI-ToF MS). Sinapic acid was used as the matrix and trifluoroacetic acid as the protonating agent. The molecular weight (M_w) of the purified enzymes was defined using MALDI-ToF MS measurement or searched from literature. Final protein purity was determined by SDS-PAGE for all the studied enzymes. Protein concentration was determined by measuring UV absorbance at 280 nm and using the molar extinction coefficients (ε) presented in Table 1.

2.3. Characterisation of the enzymes

The isoelectric point (pI) of the enzymes was determined using a horizontal electrophoresis system LBK Multiphor II (Pharmacia LKB, Sweden) according to manufacturer's instructions. A precast CleanGel polyacrylamide gel was rehydrated with a carrier ampholyte solution Pharmalyte, pH range 3–10 (GE Healthcare, UK). A broad range pI (pH 3–10) standard (GE Healthcare, UK) was used. Thermal stability of the enzymes was evaluated by measuring the melting temperatures (T_m) of enzymes using circular dichroism (CD) spectroscopy (Chirascan CD spectrometer, Applied Photophysics, UK) equipped with a CD250™ cell holder (Quantum Northwest, WA, USA) using a TC125 Peltier type temperature controller (Quantum Northwest, WA, USA). Spectra were recorded using a bandwidth of 1 nm from 240 to 190 nm with a 1 mm path length cuvette in 10 mM Na-acetate buffer pH 5 in a 3 μM enzyme concentration. Enzyme unfolding was monitored either at 202 or 220 nm using a temperature ramp program from 25 to 90 °C.

The hydrophobic surface characteristics of enzymes were evaluated using the enzyme design software Rosetta (https://www.rosettacommons.org). Large uniform hydrophobic areas on the enzyme surface were determined and given a score, namely hydrophobic patch score (Jacak et al., 2012). For the hydrophobic patch calculation, 3D protein models were obtained from the Protein Data Bank (www.rcsb.org) and proteins without structural data were modelled (Swiss-model homology modelling program) using the protein sequences obtained from Uniprot (http://www.uniprot.org/). The PDB and Uniprot codes used in this study are presented in supplementary material, Table S1. To add missing atom coordinates in the original PDB files the catalytic core of *Tr*Cel5A and CBM of *Tr*Cel7B were remodelled using the original 3D structures as templates. The 3D protein models were also used to compare enzyme surface morphology. The enzyme characteristics are presented in Table 1.