Bioresource Technology 268 (2018) 393–401

Contents lists available at ScienceDirect

Bioresource Technology

journal homepage: www.elsevier.com/locate/biortech

Comparison of catalytically non-productive adsorption of fungal proteins to lignins and pseudo-lignin using isobaric mass tagging

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ARTICLE INFO

Keywords:
Enzyme adsorption
Lignin
Pseudo-lignin
Quantitative proteomics
Isobaric mass tagging

ABSTRACT

Catalytically non-productive adsorption of fungal enzymes to pseudo-lignin (PL) was compared to adsorption to lignin preparations derived from different sources (SL, spruce; BL, birch; OL, beech) using different methods [steam pretreatment/enzymatic saccharification (SL, BL) and organosolv processing (OL)]. The protein adsorption to the SL was more extensive than the adsorption to the hardwood lignins, which was relatively similar to the adsorption to the PL. The adsorption patterns of 13 individual proteins were studied using isobaric mass tagging with TMTsixplex reagent and LC–MS/MS analysis. The results suggest that, on an average, adsorption of proteins equipped with carbohydrate-binding modules, such as the cellulases CBHI, EGII, and EGIV, was less dependent on the quality of the lignin/PL than adsorption of other proteins, such as β-Xyl, Xyn-1, and Xyn-2, which are involved in xylan degradation.

1. Introduction

Biochemical conversion of lignocellulosic biomass is one of the main pathways to bio-based fuels, chemicals, and materials (Ragauskas et al., 2006). Efficient conversion of cellulose into sugars requires pretreatment of the lignocellulosic feedstock. The most common pretreatment is hydrothermal pretreatment under acidic conditions, for example steam explosion, which targets hemicelluloses and leaves most of the cellulose and the lignin (Chandra et al., 2007; Hu and Ragauskas, 2012; Jönsson and Martin, 2016; Sun et al., 2016). Pretreatment makes enzymatic saccharification of cellulose more efficient. After enzymatic saccharification, the monosaccharides formed from cellulose and hemicelluloses can be converted to biofuels and bio-based chemicals.

Lignin is a complex polymer composed of phenylpropane units, which may be of three different types: guaiacyl (G), syringyl (S), and *p*-hydroxyphenyl (H) (Ralph et al., 2004). Softwood lignin typically consists almost exclusively of G units, whereas hardwood lignin consists of substantial fractions of both S and G units. In compositional analysis of lignocellulosic biomass, total lignin is typically reported as Klason lignin (acid-insoluble lignin) and ASL (acid-soluble lignin). During thermal pretreatment, partial decomposition of polysaccharides, such as hemicelluloses and cellulose, can result in formation of an acid-insoluble substance with aromatic characteristics known as pseudo-lignin (Brownell and Saddler, 1984; Sannigrahi et al., 2011; Normark et al., 2016). Although compositional analysis will result in inclusion of the pseudo-lignin as a part of the Klason lignin, pseudo-lignin lacks the phenylpropanoid units that are characteristic of true lignin. As hemicelluloses are more sensitive to thermal degradation than cellulose, pseudo-lignin resulting from hydrothermal pretreatment would be derived mainly from hemicelluloses.

Lignin contributes to the recalcitrance of lignocellulose by blocking the access of cellulolytic enzymes to the cellulose, by causing non-productive binding of enzymes, and by inhibition of enzymes by water-soluble phenolic compounds derived from lignin (Van Dyk and Pletschke, 2012; Kim et al., 2013; Rahikainen et al., 2013a; Vermaas et al., 2015; Zhai et al., 2016). Catalytically non-productive binding of enzymes to lignin will reduce the efficiency of enzymatic saccharification and partially prevent recycling of the enzymes.

Hydrophobic interactions between the carbohydrate-binding module (CBM) of fungal cellulases and lignin have been suggested to be the main cause of catalytically non-productive binding of cellulases to lignin (Eriksson et al., 2002; Palonen et al., 2004; Qin et al., 2014). However, some cellulases and many other hydrolytic enzymes do not have CBMs. Besides the CBM, hydrophobic areas on catalytic domains of cellulases are also believed to cause adsorption to lignin (Sammond et al., 2014). Functional groups in the lignin preparations can affect non-productive adsorption of proteins (Nakagame et al., 2011a; Pareek et al., 2013; Rahikainen et al., 2013a). For instance, the carboxylic acid content of enzyme hydrolysis residue lignin (EnzHR lignin) prepared from steam- and organosolv-treated lignocelluloses showed a negative

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https://doi.org/10.1016/j.biortech.2018.07.149
Received 1 June 2018; Received in revised form 28 July 2018; Accepted 30 July 2018
Available online 01 August 2018
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correlation with adsorption of cellulases (Nakagame et al., 2011a). The pH was also found to affect non-productive binding of cellulases to EnzHR lignin prepared from steam-pretreated spruce (Rahikainen et al., 2013b).

Cellulolytic enzymes include endoglucanases (EGs, which catalyze random cleavage of β-1,4-glucosidic bonds in the interior of cellulose chains), cellobiohydrolases (CBHs, which produce cellobiose from the chain ends of cellulose), and β-glucosidases (β-Glu, which hydrolyzes cellobiose to glucose) (Van Dyk and Pletschke, 2012). The widely studied filamentous fungus *Trichoderma reesei* (*Hypocrea jecorina*) produces many cellulase isozymes including Cel7B (EGI), Cel5A (EGII), Cel12A (EGIII), Cel61A (EGIV), Cel45A (EGV), Cel74A (EGVI), Cel61B (EGVII), Cel7A (CBHI), and Cel6A (CBHII) (Nimlos et al., 2007; Van Dyk and Pletschke, 2012). As the β-Glu activity in *T. reesei* enzyme preparations is relatively low, β-Glu from *Aspergillus niger* is usually added to supplement reaction mixtures with *T. reesei* cellulases.

Previous studies of non-productive binding of cellulose have focused mostly on the interactions of specific cellulases with lignin (Rahikainen et al., 2013b; Strobel et al., 2016; Kellock et al., 2017). Studies based on mixtures of cellulases have focused on analysis of the reduction of the total protein content or of a certain enzymatic activity (Rahikainen et al., 2011; Pareek et al., 2013), but not on individual isozymes. In industrially relevant contexts complex mixtures of enzymes and isoenzymes are typically used. To gain further insights into the problem, it is therefore important to study catalytically non-productive binding to lignins using complex systems in which different enzymes and isozymes compete with each other for the available adsorption sites of the lignin. Moreover, the relations between different properties of lignin and interactions with cellulases remain debated. For example, Ko et al. (2015) and Dien et al. (2011) offer different opinions regarding the influence of different aromatic units, especially guaiacyl (G) and syringyl (S) units, for the affinity of proteins to lignins. Kapoor et al. (2015) and Pareek et al. (2013) proposed different opinions regarding the importance of the surface area of lignins to non-productive adsorption of enzymes. Therefore, lignins from different sources and prepared using different methods need to be investigated. Furthermore, adsorption of proteins to pseudo-lignin is not well understood. It remains to be elucidated how adsorption of individual proteins to pseudo-lignin compares to the adsorption to preparations of real lignin.

The adsorption of fungal proteins to lignin and pseudo-lignin was studied using both conventional methodology, such as measurements of adsorption of total protein and enzyme activities (endoglucanase, β-glucosidase, and xylanase), and by using a novel approach based on quantitative proteomics through isobaric mass tagging and analysis with liquid chromatography tandem mass spectrometry (LC–MS/MS). Mass-spectrometry-based proteomics with stable isotope tags can offer quantitative information about proteins in complex mixtures, and permit simultaneous analysis of several different samples (reviewed by Rauniyar and Yates, 2014). Tandem Mass Tags (TMTs) are a reagent family of isotopic variants of isobaric mass tags used for labeling peptides. The isotopic variants have identical overall mass, but fragmentation during MS/MS results in formation of reporter ions that have different mass depending on which isotopic variant they belong to. TMTsixplex (Thermo Fisher Scientific, Waltham, MA, USA), which was used in the current study, is a set of six isotopic variants of TMTs that react with *N*-terminal amines and free amine groups of lysine residues of tryptic peptides.

Lignin was prepared from both hardwood and softwood, and the preparation methods included both enzymatic saccharification after steam pretreatment, and organosolv processing. Pseudo-lignin was produced from xylan through torrefaction and treatment with sulfuric acid. The lignin preparations and the pseudo-lignin were characterized by compositional analysis, Py-GC/MS (pyrolysis gas chromatography mass spectrometry), BET (Brunauer, Emmett and Teller) analysis, and FTIR (Fourier Transform Infra-Red) spectroscopy. This is the first detailed comparative study of adsorption of individual proteins to both lignin and pseudo-lignin, and the first quantitation of adsorption to lignin and pseudo-lignin of individual proteins by using isobaric mass tagging and LC–MS/MS.

2. Materials and methods

2.1. Preparation of lignin and pseudo-lignin

Enzymatic hydrolysis residue (EnzHR) lignins were prepared by enzymatic saccharification of steam-pretreated spruce and birch. Steam-pretreatment of Norway spruce (*Picea abies*) and European white birch (*Betula pubescens*) was performed in the Biorefinery Demo Plant (Örnsköldsvik, Sweden) and slurries of pretreated materials were kindly provided by SEKAB E-Technology (Örnsköldsvik). Wood chips were treated in continuous mode in a 30-L reactor. Spruce wood chips were treated as previously described (Wang et al., 2018a). Birch wood chips were treated at 190 °C for 7 min with addition of sulfur dioxide at a rate of 0.7 kg/h. The pH after pretreatment was 1.4 for spruce and 1.8 for birch. The solid residues of the spruce and birch slurries were converted to EnzHR lignin using a procedure previously described by Rahikainen et al. (2011).

Pseudo-lignin was prepared from birch wood xylan (Sigma–Aldrich, St. Louis, MO, USA), which was first torrefied in nitrogen gas at 240 °C for 20 min. The mass remaining after torrefaction (% dry weight) was 54% of the initial. Then, dilute sulfuric acid pretreatment of the torrefied xylan was performed using a single-mode microwave system (Biotage Initiator 2.0, Biotage, Uppsala, Sweden) for 165 °C and 10 min. The total reaction mixture amounted to 1000 mg, of which 50 mg was torrefied xylan, 10 mg was sulfuric acid (1% w/w), and the remainder was deionized water. The residual solids (referred to as pseudo-lignin) were collected through centrifugation (14,500*g*, 10 min), washed with deionized water, and freeze-dried.

Organosolv lignin was kindly provided by the Thünen-Institut für Holzforschung (Hamburg, Germany). It was prepared by sulfuric acid-assisted organosolv pulping of beech wood (Puls et al., 2009).

2.2. Characterization of source materials, lignin preparations, and pseudo-lignin

The steam-pretreated materials (SPS, steam-pretreated spruce; SPB, steam-pretreated birch), the lignin preparations (SL, EnzHR spruce lignin; BL, EnzHR birch lignin; OL, organosolv lignin), the material used for preparing pseudo-lignin (TX, torrefied xylan), and the pseudo-lignin (PL) were characterized. For all analyses, triplicate measurements were performed.

The mass fractions of lignin and carbohydrates were determined as previously described (Wang et al., 2018a). Py-GC/MS was used to estimate the fractions of carbohydrate and lignin, and to determine the syringyl:guaiacyl (S:G) ratio (Wang et al., 2018a).

The surface area and the porosity of the samples were determined by a single-point BET procedure by using a TriStar 3000 analyzer (Micromeritics, Atlanta, GA, USA). The BET method is based on analysis of adsorption of nitrogen gas onto the surface of a solid material. Prior to analysis, a SmartPrep Degasser (Micromeritics) was used to remove potential contaminants from the surface of the samples.

FTIR spectroscopy was performed as previously described (Wang et al., 2018b). For materials derived from xylan (TX and PL), the spectra were area normalized (wavenumber range 1800–400 cm^{-1}).

For checking protein adsorption to EnzHR lignin preparations owing to the protease treatment, the nitrogen contents of steam-pretreated, enzymatically hydrolyzed, and protease-treated spruce and birch were analyzed by Bränslelaboratoriet (Umeå, Sweden). The analysis was based on ISO method 16948 (2015).

The contact angles of water droplets on films of lignin or PL were measured by MoRe Research (Örnsköldsvik, Sweden) by using a DAT 1100 Dynamic Absorption Tester (Fibro System AB, Stockholm,

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