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Adsorption of proteins involved in hydrolysis of lignocellulose on lignins and hemicelluloses

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HIGHLIGHTS

- Unproductive enzyme adsorption on lignocelluloses decreases bioconversion rate.
- Adsorption of enzymes to eight lignocellulosic substances was investigated.
- BET, FTIR & GPC were used to characterize lignins used in adsorption experiments.
- Xylan and mannan adsorbed a significant fraction (>15%) of the cellulase protein.
- Lignins adsorbed more protein, and hydrophobic interactions play an important role.

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ABSTRACT

Protein adsorption onto eight lignocellulosic substances (six lignin preparations and two hemicelluloses) was investigated at pH 4.8 and at two different temperatures (4 °C and 45 °C). The kinetics of the adsorption of cellulase, xylanase, and β-glucosidase were determined by enzyme activity measurements. The maximum adsorption capacities, the affinity constants and the binding strengths varied widely and were typically higher for the lignins than for the carbohydrates. As indicated by BET and gel permeation chromatography, different substances had widely different surface area, pore size, weight average molecular weight, and polydispersity index, but these properties were difficult to relate to protein binding. In most cases, an increase in temperature from 4 °C to 45 °C and a low content of carboxylic acid groups, as indicated by Fourier-Transform Infra-Red (FTIR) spectroscopy, resulted in increased protein adsorption capacity, which suggests that hydrophobic interactions play an important role.

1. Introduction

Conversion of lignocellulosic biomass to biofuels and chemicals offers decreased dependency on fossil sources, improved energy security, and a move from environmental challenges associated with petroleum. Two major constituents of lignocellulosic biomass, cellulose and hemicellulose, can be hydrolyzed to monosaccharide sugars that can be utilized for production of bioalcohols, carboxylic acid and other commodities in microbial fermentation processes. The third main component, lignin, contributes to the recalcitrance of lignocellulosic feedstocks by making enzymatic hydrolysis difficult by unproductively adsorbing hydrolytic enzymes, such as cellulases and β-glucosidases, thereby reducing the availability of free enzymes. Enzyme recycling, which would be considered as a good strategy to render the process more cost-effective, is made difficult by enzyme adsorption to the lignin-rich residue (Palonen et al., 2004; Berlin et al., 2005, 2006; Tu et al., 2007a,b; Varnai et al., 2010; Nakagame et al., 2010).

Pretreatment makes the recalcitrant lignocellulosic biomass more susceptible to hydrolytic enzymes. Pretreatment affects enzyme adsorption to the substrate via altering its surface properties (Wyman et al., 2005; Chandra et al., 2007). Enzyme adsorption significantly varies with the biomass source. The surface properties of both the protein and the lignocellulosic substrate affect the intensity of hydrophobic, electrostatic, and other interactions, and hence the adsorption processes (Haynes and Norde, 1994). Adsorption of cellulases to isolated lignins as well as lignocellulose has been studied (Tu et al., 2009a; Nakagame et al., 2011a,b; Pribowo et al., 2012). Most of the studies have involved lignins extracted from the same biomass source and pretreated under similar condi-

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tions. In order to understand the fundamentals behind protein adsorption, it is of interest to compare the adsorption capacities of lignins derived from diverse types of biomass pretreated by different methods, and to take their physico-chemical properties into account. Also, it would be of interest to study total protein adsorption and mono-component enzyme adsorption using the same substances. Furthermore, most of the adsorption studies have been focused on cellulases while adsorption of β-glucosidase and xylanase is less well studied. Apart from the lignins, hemicelluloses are also present in notable amounts in lignocellulosic biomass and may potentially adsorb enzymes. So far, relatively little work has been performed on the adsorption of cellulases and β-glucosidases on hemicelluloses.

The present study focus on adsorption of two important commercial enzyme preparations, Celluclast 1.5L and Novozyme 188, on lignin preparations originating from different types of processes (kraft pulping, sulfite pulping, organosolv pulping and hydrolysis) and biomass sources (softwood and hardwood), as well as on two different types of hemicelluloses (xylan and galactomannan). Variations in surface properties, molecular weight and chemical functionalities of lignins were determined in order to investigate potential connections to enzyme adsorption efficiency. Temperature dependence of adsorption was studied by monitoring the adsorption profiles of the enzymes at two different temperatures (4 °C and 45 °C). Variations in the adsorption kinetics of the enzymes were evaluated by quantifying total protein content as well as individual enzyme components.

2. Methods

2.1. Polymeric substances and enzyme preparations

Slurries of pretreated Norway spruce (*Picea abies*) and black cottonwood (*Populus trichocarpa*) were utilized to prepare softwood and hardwood lignin, respectively. Pretreatments were performed by SEKAB (Örnsköldsvik, Sweden) in the biorefinery demonstration plant in Örnsköldsvik. The black cottonwood was treated in a continuous mode at a temperature of 190 °C (pressure, 13 bar) in a 30-L reactor and with a residence time of 6–7 min. The feed rate of the raw material was 50 kg/h (dry weight) and the wood chips were impregnated with sulfur dioxide (0.5 kg/h). The pH after the pretreatment was 2.7. The pretreatment of Norway spruce (*P. abies*) was also performed in continuous mode and the conditions were: temperature, 205 °C; pressure, 18 bar; feed rate, 40 kg/h of raw material; impregnation with sulfur dioxide (1.0 kg/h); residence time in reactor, 7–8 min. The pH after pretreatment was 1.6. The pretreated material was cooled and stored at 4 °C until further use.

Lignin preparations [viz. alkali lignin (AL), hydrolytic lignin (HL), organosolv lignin (OL), and lignosulphonic acid sodium salt (LS)], beech wood xylan (BWX), and galactomannan (GM) (Locust bean gum) were procured from Sigma–Aldrich (St. Louis, MO, USA). The two enzyme preparations used in the study, Celluclast 1.5L (700 EGU g^{-1}; protein content 40 mg mL^{-1}) and Novozyme 188 (250 CBU g^{-1}; protein content 51 mg mL^{-1}), were also obtained from Sigma–Aldrich. Celluclast 1.5L, which is derived from *Trichoderma reesei* ATCC 26921, served as the main source of cellulase and xylanase enzymes, while Novozyme 188, which is derived from *Aspergillus niger*, was used as the main source of β-glucosidase.

2.2. Lignin extraction

Lignin was isolated from pretreated lignocellulose employing a two-step sulfuric acid treatment (Laboratory Analytical Procedure LAP-003, National Renewable Energy Laboratory, Golden, CO). Briefly, pretreated lignocellulose was hydrolyzed with sulfuric acid (72% w/w, 30 °C, 2 h) followed by autoclaving at 121 °C for 1 h. Prior to the second step samples were diluted to an acid concentration of 4% (w/w). After the second hydrolysis step, the samples were cooled down and solids were collected by filtering through a 1.6 μm glass fiber filter (Whatman, Maidstone, UK). Solid fractions, referred to as SL (spruce lignin) and PL (*Populus* lignin), were washed with water (pH adjusted to 2.5 with HCl), freeze-dried, and stored at room temperature.

2.3. Lignin characterization

The surface area and the pore size of the lignin preparations were determined by single-point BET (Brunauer–Emmett–Teller) procedure using a Tristar surface area and porosity analyzer (Micromeritics, Norcross, GA). The BET method is based on nitrogen adsorption onto the polymer surface at different pressures. Prior to analysis, the lignins extracted from pretreated spruce and cottonwood slurries were dried overnight at 80 °C while no further drying was performed for the commercial lignin samples.

The weight average molecular weight (M_w), number average molecular weight (M_n) and polydispersity index (PDI, M_w/M_n) of the lignin preparations were determined by gel permeation chromatography (GPC). The GPC separation system consisted of a Polymer Laboratories PL-GPC 50 Plus instrument with an ultraviolet (UV) detector (254 nm), a 50 × 7.5 mm PolarGel guard column, and two 300 × 7.5 mm PolarGel M columns (Agilent, Santa Clara, CA). A 1:2 (v/v) H_2O:dimethylacetamide (DMAc) mixture containing 0.1% (w/w) lithium bromide (LiBr) was used as eluent. The eluent flow-rate was set to 0.5 mL min^{-1}. The system was calibrated using 12 sodium polystyrene sulfonate (PSS) standards ranging from 208 g mol^{-1} to 2.6×10^6 g mol^{-1}. Lignin preparations (AL, HL, OL, and LS) were dissolved in DMAc and LiBr (0.1% w/w) at concentrations of approximately 1 mg mL^{-1} and passed through a 0.45 μm filter prior to analysis. LS and AL were easier to dissolve than HL and OL, while SL and PL did not dissolve in the DMAc–LiBr system.

The chemical groups of the isolated lignins were analyzed using Fourier Transform Infra-Red Spectroscopy (FTIR) performed with an IFS 66v/S vacuum spectrometer (Bruker, Billerica, MA) with a standard Deuterated Triglycine Sulfate detector, and fitted with a diffuse reflectance accessory (Bruker Corporation), by averaging 64 scans over the range 400–4000 cm^{-1}. Sample powders were ground together with potassium bromide (KBr) and filled in an FTIR sample cup holder. The spectra were baseline-corrected and analyzed with the OPUS 5.0 software (Bruker). KBr was spectrograde and purchased from Thermo Fisher Scientific (Waltham, MA).

2.4. Enzyme adsorption

The competitive adsorption of the two enzyme preparations, viz. Celluclast 1.5L and Novozyme 188, onto the lignins and the polysaccharides was performed in polypropylene tubes (2 mL) using 1% (w/v) lignocellulosic substance and 10 mg mL^{-1} of protein in 50 mM sodium acetate buffer (pH 4.8). With regard to the lignin preparations, most of them (SL, PL, HL and OL) appeared insoluble, while the others showed some solubility. In order to study the effect of temperature on the enzyme adsorption, incubations of reaction mixtures were carried out for 72 h at both 4 °C and 45 °C in a rotary shaker. Aliquots withdrawn at various time intervals were centrifuged (10,000*g*, 10 min) and the liquid containing unbound enzymes was collected (adsorption supernatant) for further analysis. Controls lacking lignin, polysaccharides, or enzymes were used as reference.

2.5. Adsorption isotherm

The adsorption of the two enzyme preparations to the lignins and the polysaccharides was studied by using the Langmuir isotherm. Various concentrations (2–40 mg/mL) of protein were incubated with the different substances (lignin or polysaccharide) suspended in acetate buffer (50 mM, pH 4.8). The adsorption parameters, viz. the maximum adsorption capacity (σ) and the equilibrium constant (K_d), were determined by non-linear regression of the adsorption data using Eq. (1), where [*CE*] represents adsorbed enzyme in mg/mL, [E_f] the free enzyme concentration in mg/mL, σ the maximum adsorption capacity in mg/mg substrate, [S_t] the substrate concentration in mg/mL, and K_d denotes the equilibrium constant ([*C*][*E*]/[*CE*]) in mg of enzyme/mL:

$$[CE] = \sigma[S_t][E_f]/K_d + [E_f] \quad (1)$$

The affinity constant ($A = 1/K_d$) and the binding strength ($S = A \times \sigma$) were also analyzed.

2.6. Analytical methods

2.6.1. Protein assay

The protein content in the supernatant was measured according to Bradford (1976) using bovine serum albumin (BSA) as the standard. All the measurements were done in triplicates and the average values are presented.

2.6.2. Enzyme activity assays

The endoglucanase activity was measured using carboxymethyl-cellulose (CMC, 1%) as substrate in 0.05 M acetate buffer (pH 4.8) incubated at 50 °C for 30 min. Activity measurements for xylanase were carried out using beech wood xylan (1%) as substrate. The reducing sugars released from CMC and xylan were determined using dinitrosalicylic acid reagent employing calibration with glucose and xylose, respectively. One unit of enzyme activity corresponds to one μmol of sugar liberated per min under standard assay conditions.

The β-glucosidase activity was measured using 1 mM 4-nitrophenyl-β-D-glucopyranoside (PNPG) as the substrate. Briefly, a solution of the substrate (1 mL) was mixed with 0.1 mL of diluted enzyme solution and incubated for 10 min at 50 °C. The reaction was terminated by addition of 1 M Na_2CO_3 (2 mL) and diluted with 10 mL distilled water. The amount of liberated 4-nitrophenol was measured at 400 nm.

3. Results and discussion

3.1. Protein adsorption kinetics

Enzyme adsorption was studied using lignin preparations derived from different types of processes (AL, HL, OL, and LS) and different origin, such as softwood (SL, spruce lignin) and hardwood (PL, *Populus* lignin). In addition, two hemicelluloses were included in the investigation (BWX and GM). The adsorption profiles of two enzyme preparations, Celluclast 1.5L and Novozyme 188, onto lignins and polysaccharides were followed during a period of 72 h. The adsorption capacity of the substances investigated varied considerably. The lignin preparations generally adsorbed protein more efficiently than the polysaccharides (Figs. 1 and 2). At 4 °C, the highest apparent adsorption values were obtained with lignosulfonate (74% for Celluclast 1.5L and 59% for Novozyme 188) followed by alkali lignin (72% for Celluclast 1.5L and 53% for Novozyme 188). At 4 °C, lignin derived from pretreated spruce adsorbed more protein (43% for Celluclast 1.5L and 44% for Novozyme 188) than black cottonwood lignin (26% for Celluclast 1.5L and 37% for Novozyme

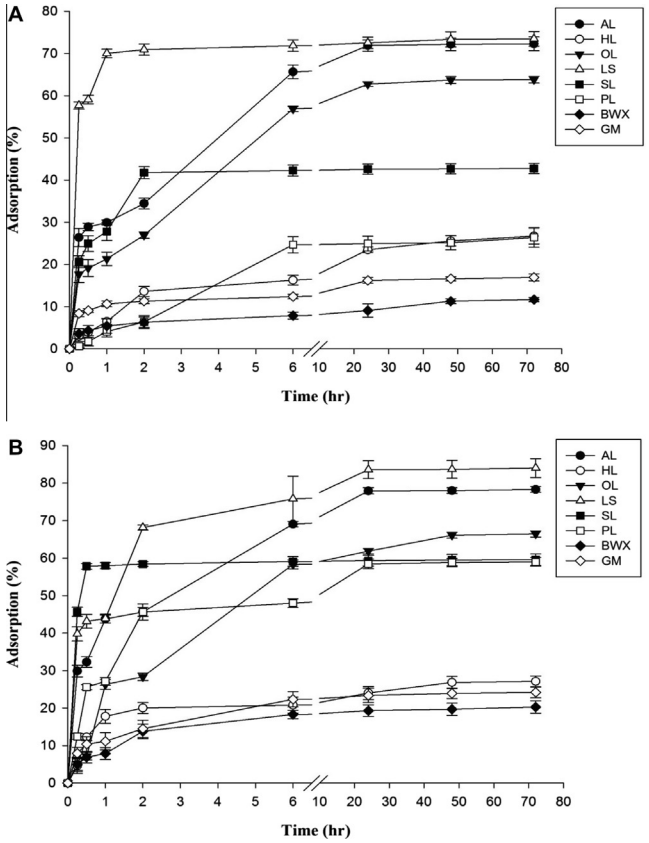

Fig. 1. Adsorption kinetics of Celluclast 1.5L at (A) 4 °C and (B) 45 °C. AL, alkali lignin; HL, hydrolytic lignin; OL, organosolv lignin; LS, lignosulfonate; SL, spruce lignin; PL, *Populus* lignin; BWX, beech wood xylan; GM, galactomannan. Adsorption experiments were performed using 1% (w/v) of each polymeric substance tested and 10 mg protein mL^{-1} of final reaction mixture in 50 mM sodium acetate buffer (pH 4.8). Values are expressed as the mean ± SD (error bars) of three independent experiments.

188) (Figs. 1 and 2). At 45 °C, spruce and black cottonwood lignin showed similar maximum adsorption, but their time-dependent adsorption profiles differed (Figs. 1 and 2). Protein adsorption to spruce lignin was faster as more than 50% of the protein had adsorbed after 30 min of incubation, while it took more than 2 h for the black cottonwood lignin to reach the same value (Fig. 1b). The difference between spruce and black cottonwood lignin was most obvious for Celluclast 1.5L at 4 °C. With regard to the polysaccharides, galactomannan always showed slightly higher adsorption efficiency than xylan (Figs. 1 and 2).

Time course studies of enzyme adsorption were conducted to investigate the time when equilibrium was reached. Equilibrium for adsorption was reached after 2 h, hence this time period was chosen for further adsorption studies (Figs. 1 and 2). The time period required to attain equilibrium during adsorption varies according to the biomass, the protein, and the incubation conditions. Cellulolytic enzyme lignin from steam-exploded and ethanol-pretreated lodgepole pine was found to require 3 h to reach equilibrium during adsorption of cellulase preparations from *T. reesei* and *Penicillium* sp. (Tu et al., 2009a). Lignin extracted from pretreated Douglas fir also attained adsorption equilibrium in 3 h (Nakagame et al., 2011b). Cellulase adsorption to corn stover solids required less than 2 h (90 min) to attain equilibrium (Lynd et al., 2002; Kumar and Wyman, 2009).

The effect of temperature on enzyme adsorption was evaluated by performing the adsorption experiments at 4 °C and 45 °C. Similar adsorption profiles were observed at both temperatures, but generally the fraction of protein that adsorbed to the substances

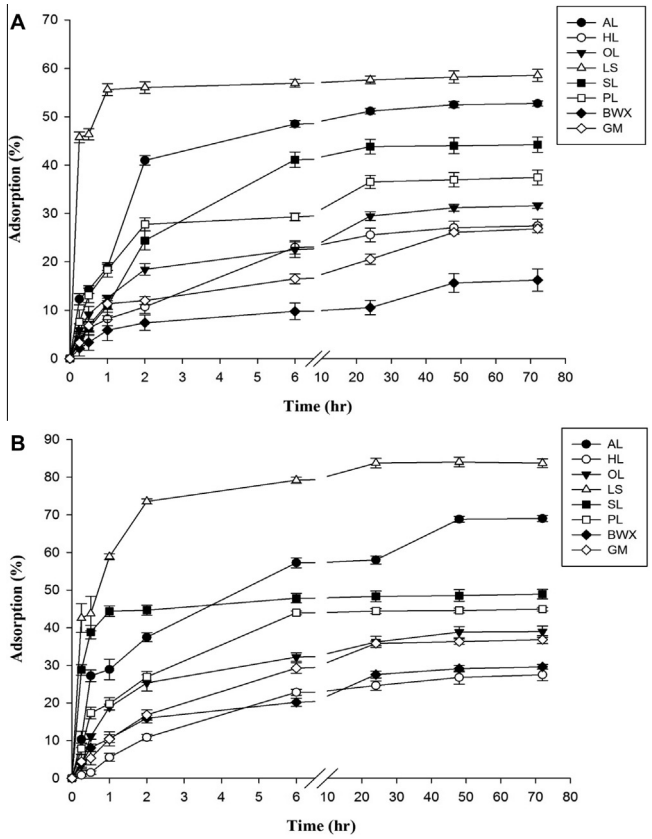

Fig. 2. Adsorption kinetics of Novozyme 188 at (A) 4 °C and (B) 45 °C. Values are expressed as the mean ± SD (error bars) of three independent experiments.

Table 1
Adsorption parameters of enzyme preparations at 4 °C and 72 h.[a,b]

Substance	Maximum adsorption capacity (mg protein g^{-1} substrate)	Affinity constant (A) (L g^{-1} protein)	Binding strength (S) (mL g^{-1} substrate)	R^2
A. Enzyme preparation Celluclast 1.5L				
AL	113	2.5	282	0.97
HL	50	1.7	84	0.95
OL	94	2.3	214	0.97
LS	127	2.9	365	0.97
SL	117	1.7	195	0.98
PL	141	1.2	169	0.99
BWX	44	1.1	48	0.91
GM	49	1.3	65	0.97
B. Enzyme preparation Novozyme 188				
AL	114	2.0	232	0.99
HL	44	2.1	93	0.97
OL	68	2.5	169	0.97
LS	123	3.0	362	0.96
SL	82	1.9	158	0.98
PL	91	1.3	123	0.97
BWX	47	1.1	54	0.95
GM	53	1.4	75	0.98

[a] Experiments were performed in triplicates and the mean values are presented.
[b] AL, alkali lignin; HL, hydrolytic lignin; OL, organosolv lignin; LS, lignosulfonate; SL, spruce lignin; PL, *Populus* lignin; BWX, beech wood xylan; GM, galactomannan.

was higher at 45 °C than at 4 °C (Figs. 1 and 2). Protein adsorption on the surface of lignin is considered to be an endothermic process. The increase in adsorption with corresponding increase in temperature might be due to increased hydrophobic interactions, which are believed to play an important role in protein adsorption onto lignin. An increase in temperature may also affect the molecular structure of proteins leading to exposure of more hydrophobic regions to the surroundings and hence enhanced hydrophobic interactions (Bonomo et al., 2006). Although hydrophobic interactions are considered as the main contributor to the adsorption process, electrostatic forces also play an important role. Apart from catalytically unproductive enzyme adsorption, lignin contributes to the inhibition of hydrolysis through limiting the accessible surface area of cellulose by restricting swelling and via deposition of small spherical lignin droplets on the fiber surface, i.e. through a shielding effect (Donohoe et al., 2008).

With the spruce lignin, the effect of the temperature was more pronounced as the adsorption equilibrium was attained in a shorter period of time (30 min) with both protein preparations. (Figs. 1b and 2b). While most substances adsorbed more protein at 45 °C than at 4 °C, this was less evident for HL (Figs. 1 and 2). Hydrophobic interactions may therefore have been comparatively less important for the capability of HL to bind protein than for the other lignin preparations. Temperature has been observed to impose variable effects on enzyme adsorption on lignins. In some cases changes of the temperature lead to increased or decreased protein adsorption (Creagh et al., 1996; Tu et al., 2009a,b; Rahikainen et al., 2011), while in other cases no significant changes in the adsorption were detected with temperature (Piccolo et al., 2010). Adsorption of Celluclast 1.5L on lignin extracted from pretreated spruce has been observed to increase at 45 °C (Rahikainen et al., 2011). Similarly, the inhibitory effect of lignin extracted from spruce and wheat straw on three cellobiohydrolases, viz. *Tr*Cel7A, *Te*Cel7A-CBM1 and *Te*Cel7ACBM3, was found to increase with the temperature due to enhanced adsorption (Rahikainen et al., 2013). A change in temperature from 4 °C to 50 °C has been reported to result in a tenfold enhancement of the capacity of lignin to adsorb cellulase (Zheng et al., 2013). The slow kinetics observed during the adsorption was attributed to a phase transition related to the porous structure of the solid material.

Novozyme 188 had similar adsorption profiles as Celluclast 1.5L but was adsorbed to a lesser degree onto the polymeric substances analyzed (Fig. 2). For instance, the maximum adsorption capacity of SL and PL was much lower for Novozyme 188 than for Celluclast 1.5L. The adsorption proceeded relatively slowly and the equilibrium was typically attained following 6 h (Fig. 2). This might be due to the absence of CBMs (carbohydrate-binding modules) in the key enzyme of the preparation, i.e. β-glucosidase, since CBMs are expected to play a critical role in adsorption of proteins onto lignins. In most cases, the relative order of the capacity of the different substances to adsorb protein was the same for Celluclast 1.5L (Fig. 1) and Novozyme 188 (Fig. 2), with LS and AL showing the highest and the second highest final apparent adsorption values, respectively. However, OL showed the third highest adsorption capacity for Celluclast 1.5L (Fig. 1), but only the fifth highest adsorption capacity for Novozyme 188 (Fig. 2). This suggests that

Table 2
Surface area and pore-size determination by BET analysis.[a]

Polymeric substance	Surface area (m^2/g)	Pore size (nm)
Alkali lignin	1.2	50
Hydrolytic lignin	4.6	21
Organosolv lignin	0.9	13
Lignosulfonic acid	n.d.[b]	n.d.
Spruce lignin	2.1	190
Populus lignin	1.0	17
Beech wood xylan	2.5	39
Galactomannan	0.2	73

[a] Data presented are mean values of two independent measurements.
[b] Not determined.

Table 3
Analysis of lignin preparations using Gel Permeation Chromatography (GPC).[a]

Lignin	M_n	M_w	PDI
Alkali lignin	500	3100	6.2
Hydrolytic lignin	700	6500	9.8
Organosolv lignin	n.d.[b]	700	n.d.
Lignosulfonic acid	2400	13,400	5.6

[a] Data presented are mean values of three independent measurements.
[b] Not determined.

cellulase binding and the presence of CBMs were especially prominent in the interactions with OL.

The protein adsorption efficiency of different lignin preparations has been found to be significantly affected by its isolation method (Sutcliffe and Saddler, 1986). Lignin extracted from steam-pretreated spruce using acid hydrolysis has been observed to have higher protein-binding capacity and more inhibitory effect on Avicel hydrolysis via non-productive binding of cellulases as compared to the enzymatic hydrolysis residue. Acid utilized during the extraction process affects the adsorption capacity of lignins primarily via altering the surface chemistry and secondly by increasing the available surface area for enzyme binding (Rahikainen et al., 2011). Our findings (Figs. 1 and 2) are in good agreement with these observations. Commercial lignin preparations from different sources and processes can have different physical properties as well as chemical functionalities, which would affect their enzyme adsorption efficiency.

3.2. Langmuir adsorption isotherms of protein to lignin

During enzymatic hydrolysis, the non-productive binding of cellulases to lignin renders the enzyme unavailable for catalysis and this also causes problems for enzyme recycling. To gain an insight about the enzyme adsorption to lignin, the maximum adsorption capacity, affinity and binding strength were estimated following incubation at 4 °C employing the Langmuir isotherm. The adsorption data for both the enzyme preparations fitted well with the Langmuir isotherm (Table 1).

Among the lignin samples analyzed, the black cottonwood lignin had the highest adsorption capacity (141 mg protein g^{-1} lignin) for Celluclast 1.5L. The adsorption capacity decreased in the order PL > LS > SL > AL > OL > HL > GM > BWX (Table 1a). The highest apparent affinity and binding strength was observed for lignosulfonate (2.9 L g^{-1} protein, 365 mL g^{-1} protein) followed by alkali lignin (2.5 L g^{-1} protein, 282 mL g^{-1} protein). The hydrolytic lignin had the lowest adsorption capacity among the lignin preparations studied. The variations in the adsorption parameters for the commercial lignin samples might be due to the different processing methods. Lignin extracted from pretreated black cottonwood had higher protein adsorption capacity but lower affinity and binding strength than the spruce lignin. Higher affinity values suggest that the lignin extracted from spruce had more adsorption sites. The recalcitrance of softwood biomass to enzymatic hydrolysis can, at least in part, be attributed to the high affinity of softwood lignin to proteins in hydrolytic enzyme mixtures.

For Novozyme 188, the apparent adsorption capacity of lignosulfonate (123 mg protein g^{-1} lignin) was considerably higher than that of the other substances. Also here the lignin extracted from black cottonwood had higher adsorption capacity than the spruce lignin, but the latter had higher values with regard to the affinity constant and the binding strength. The beech wood xylan and the galactomannan showed slightly higher values for the adsorption parameters than was observed for corresponding experiments with Celluclast 1.5L (Table 1). The maximum adsorption capacity and binding strength of HL were closer to the range exhibited by the hemicelluloses than to those of the other lignin preparations (Table 1). The maximum adsorption capacities observed for lignin from pretreated spruce and black cottonwood (Table 1) were higher than reported for lignins from corn stover, lodgepole pine, poplar and Douglas fir (Kumar and Wyman, 2009; Nakagame et al., 2011a,b).

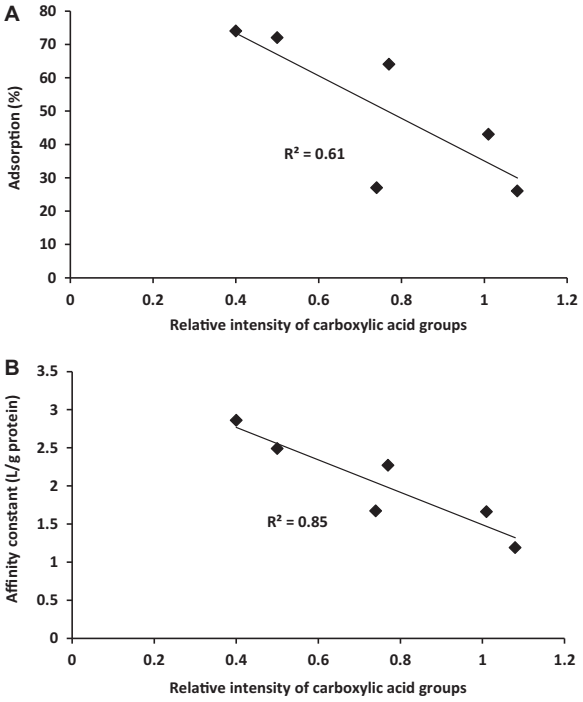

Fig. 3. Correlation of (A) the adsorption (%) of Celluclast 1.5L and (B) the adsorption affinity of lignins with the relative intensity of carboxylic acid groups as determined by using Fourier-Transform Infra-Red (FTIR) spectroscopy.

3.3. Surface analysis

The variations in the adsorption properties would be affected by the method of preparation, which in turn affects the physical and chemical properties of the lignin. Therefore, it is important to determine how the chemical functionalities and physical properties influence protein binding to lignin.

Surface area and pore size of lignins, xylan and mannan were determined employing BET analysis, since adsorption of protein to polymers largely depends on the surface properties and is the first step during enzymatic hydrolysis. The surface properties of the lignin are influenced by the biomass source and the pretreat-

Table 4
Analysis of lignin preparations using Fourier-Transform Infra-Red (FTIR) spectroscopy.[a,b]

Functional group	cm^{-1}	AL	HL	OL	LS	SL	PL
Carbonyl groups (C=O)	1750–1600	1.18	0.87	0.82	0.96	1.03	1.05
Carboxylic acid groups (HO–C=O)	1715–1700	0.50	0.74	0.77	0.40	1.01	1.08
Ester linkages (O=C–O)	1235–1210	0.86	0.97	1.03	1.14	2.75	3.29
Ether linkages (–O–)	1140–1110	1.01	0.99	1.04	0.94	1.44	1.52

[a] The relative intensity of the chemical groups to the intensity of 1500 cm^{-1}, i.e. aromatic skeletal vibrations (C=C) in lignin.
[b] AL, alkali lignin; HL, hydrolytic lignin; OL, organosolv lignin; LS, lignosulfonate; SL, spruce lignin; PL, *Populus* lignin.

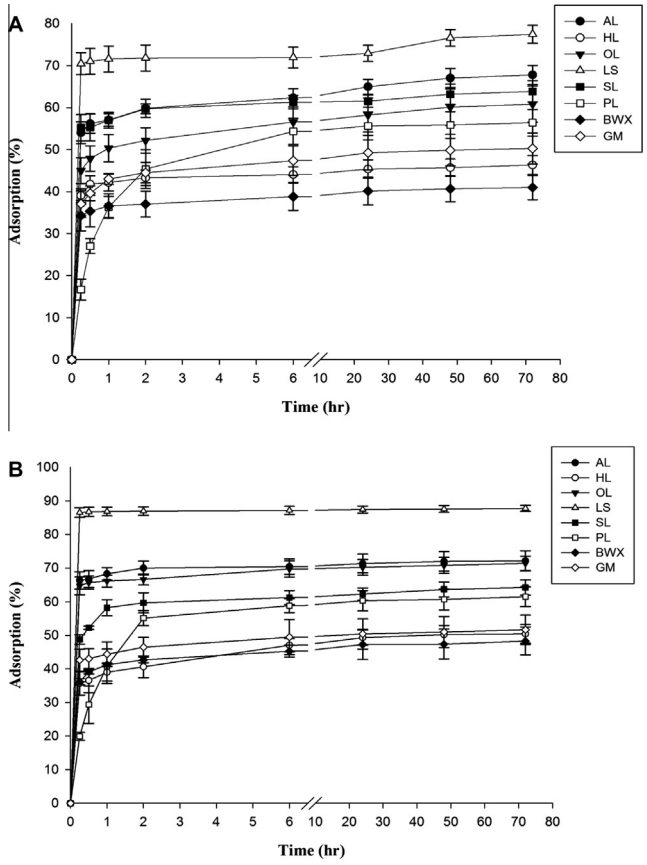

Fig. 4. Time course profiles of endoglucanase adsorption at (A) 4 °C and (B) 45 °C. Values are expressed as the mean ± SD (error bars) of three independent experiments.

ment conditions. The specific surface area values ranged between 0.2 and 4.6 m^2/g for the samples analyzed (Table 2). Large variations were observed with regard to the pore size of the various substances (13–190 nm). Lignin extracted from pretreated spruce had higher specific surface area and pore size than the black cottonwood lignin (Table 1), which may explain its rapid protein adsorption. The maximum adsorption capacities of lignins isolated from steam and organosolv pretreated corn stover, poplar and lodgepole pine did not correlate with the observed specific areas of the lignins (Nakagame et al., 2011a). Similarly, in the present investigation, the maximum protein adsorption capacity of the commercial lignin preparations and the hemicelluloses did not correlate with the observed specific surface area and pore size (Table 2). The surface area of lignins has been observed to increase with the pretreatment severity of spruce, wheat straw (Piccolo et al., 2010) and Douglas fir (Nakagame et al., 2011b) and have negative effects on enzymatic hydrolysis. Other studies suggest that the initial rate of hydrolysis shows a positive correlation with the available surface area (Sinitsyn et al., 1991; Thompson et al., 1992; Chandra et al., 2008).

3.4. Molecular weight determination

The average molecular weight (M_w and M_n) of the commercial lignin preparations was determined by using GPC (Table 3). Several different solvent systems have been reported for dissolution of lignin from woody plants, but the acid-insoluble lignins extracted in the present study (SL and PL) did not dissolve in any of the solvent systems tested (DMAc–LiBr, dioxane, 1,4-butanediol, dimethyl sulphoxide, toluene, cyclohexane, ethyl acetate). The conditions during analytical procedures, such as BET analysis and GPC, differ from the conditions used during protein adsorption experiments, which can affect the properties of the polymers studied. These analytical methods can, however, provide relevant information about the characteristics of the different lignin preparations included in the study.

The average M_w and M_n values for lignosulfonate were larger than those of alkali lignin and hydrolytic lignin (Table 3). Organosolv lignin had a M_w of only 700, much less than any of the other lignin preparations, which indicates that it was heavily degraded. Previous studies have indicated that the polydispersity index (PDI) was inversely related to the interaction of the polymer with the protein (Berlin et al., 2006). The low polydispersity values of lignosulfonate and alkali lignin (Table 3) would seem to agree with the above hypothesis, since these two lignin preparations had the highest apparent protein adsorption efficiency (Figs. 1 and 2). In line with that, the hydrolytic lignin, which had a high degree of polydispersity, exhibited low protein adsorption. However, protein adsorption by lignin extracted from corn stover, poplar and lodgepole pine was not found to be related to the polydispersity value (Nakagame et al., 2011a).

3.5. Spectroscopic analysis

The chemical structure of lignins was studied in order to investigate if there was a correlation between the relative amounts of various chemical functionalities within lignin and the protein adsorption efficiency. The relative amounts of the chemical groups in the lignins (Table 4) were determined by calculating the ratio of the intensity of the different bands in comparison to the intensity of the band at around 1500 cm^{-1}, originating from aromatic skeletal vibrations (C=C) in lignin (Faix, 1991).

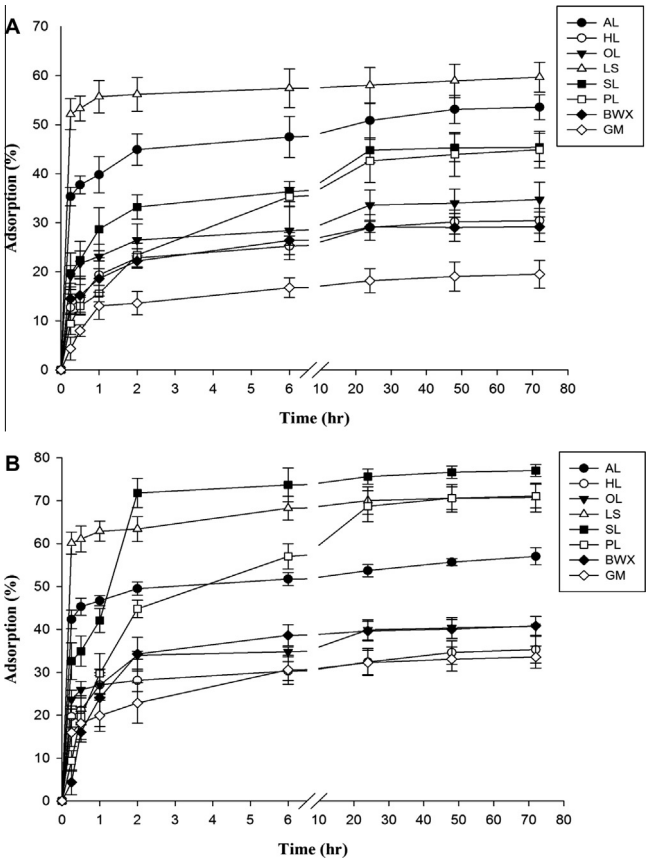

Fig. 5. Time course profiles of xylanase adsorption at (A) 4 °C and (B) 45 °C. Values are expressed as the mean ± SD (error bars) of three independent experiments.

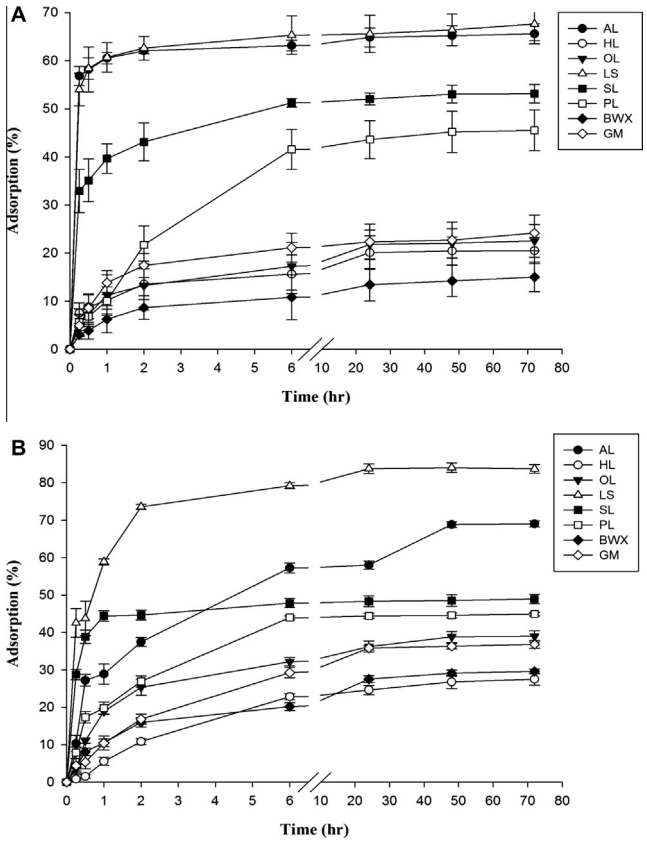

Fig. 6. Time course profiles of β-glucosidase adsorption at (A) 4 °C and (B) 45 °C. Values are expressed as the mean ± SD (error bars) of three independent experiments.

The relative intensity of the carboxylic acid groups in the lignin preparations appeared to correlate with protein adsorption (Fig. 3a) and adsorption affinity constants (Fig. 3b). However, the lignosulfonates do not fit into this comparison, as they are more charged and more hydrophilic than any other of the lignin preparations due to their sulfonate groups. Thus, the correlation observed for lignosulfonates in Fig. 3 is only apparent. The explanation why lignosulfonates remove protein more efficiently than the other lignin preparations may possibly be their capability to precipitate protein (Cerbulis, 1978; Xu et al., 2001). In that case, the mechanism by which lignosulfonates remove protein differs from that of the other substances and comparisons are difficult to make. The correlation between carboxylic acid content and low protein adsorption agrees with previous findings, which showed that the hydrolysis yield of Avicel exhibited a positive correlation with the carboxylic acid content of lignin (Nakagame et al., 2011a). A possible explanation for this effect is that the increase in carboxylic acid content will increase the hydrophilicity of the lignin, which in turn negatively affects the non-productive binding of the cellulases to lignin during hydrolysis.

3.6. Enzyme quantification studies

Protein quantification gives an overview of the enzyme adsorption capacities of the lignin preparations and the hemicelluloses. In order to gain an insight about adsorption of prominent enzymes in the protein preparations studied, activity assays for endoglucanase (cellulase), xylanase and β-glucosidase were carried out.

Analysis of the supernatants remaining after experiments performed at 4 °C and 45 °C revealed that all the substances studied decreased the cellulase activity (Fig. 4). After incubation with lignosulfonate at 4 °C, only 30% of the activity remained in the supernatant (Fig. 4a). Alkali lignin, organosolv lignin and spruce lignin showed similar levels of adsorption (61–68%). The protein adsorption values of organosolv lignin were the third or fourth highest in the series (Fig. 4), in agreement with results with total protein of Celluclast 1.5L (Fig. 1). Among the lignin preparations studied, the hydrolytic lignin adsorbed the lowest amount of cellulase activity (46%) following 72 h of incubation (Fig. 4a). The enzyme adsorption values observed for xylan and mannan (~50%) were lower than for most of the lignin preparations. A notable increase in cellulase adsorption was detected when the temperature was raised to 45 °C (Fig. 4b), which indicates the importance of hydrophobic interactions. Rahikainen et al. (2011) showed that the lignin-rich residues (obtained via acid and enzymatic hydrolysis) and cellulose-rich substrates, such as Avicel and steam-pretreated spruce, exhibit different adsorption patterns. The lignin obtained from acid hydrolysis adsorbed 90% of the total endoglucanase activity, whereas 60% activity was adsorbed on lignin derived from enzymatic hydrolysis (Rahikainen et al., 2011). Cellulases (TrCel7A and TrCel5A) were observed to bind extensively (more than 98%) onto steam-pretreated and catalytically delignified spruce (Varnai et al., 2011).

Xylanase was adsorbed to lesser degree than cellulase, but followed a similar adsorption pattern (Fig. 5). Furthermore, the initial adsorption of xylanase was rather slow, which partially might be due to the absence of CBMs. The effect of temperature was very evident with regard to adsorption of xylanase to spruce and black cottonwood lignins (Fig. 5b), and the highest value was obtained for spruce, which adsorbed 77% of the activity at 45 °C. The lignin preparations adsorbed more xylanase than xylan (Fig. 5). These results agree with those of Varnai et al. (2011), who reported that the lignin content of the substrate had more influence on xylanase adsorption than the xylan content, and that steam-pretreated spruce with low xylan content adsorbed much of the xylanase activity. With regard to the hemicelluloses, the adsorption of xylanase at 4 °C was higher for xylan than for glucomannan (Fig. 5a). This was the only case where the adsorption of xylan appeared higher than that of glucomannan, which can be attributed to the preference of xylanase for its native substrate.

Novozyme 188 is typically added to hydrolysis reactions as a source of β-glucosidase. Assay of β-glucosidase activity in the supernatants revealed that it was adsorbed to the substances to a lesser degree than cellulase (Fig. 6). This can be attributed to the lack of CBM of β-glucosidase. After 72 h of incubation, the lowest amount of free β-glucosidase activity was found in the lignosulfonate sample. Lignins adsorbed higher levels of enzyme than the hemicelluloses, but notable enzyme adsorption was detected also for xylan and mannan (15–30%). Similar results were obtained when the total protein adsorption of Novozyme 188 was studied (Fig. 2). There are contradictory results in the literature regarding β-glucosidase adsorption. This might be due to the structural and compositional differences between the polymeric substances used in different studies, specifically the different structures of lignins, and it might also be due to differences in the experimental set-up. Comparatively less adsorption of β-glucosidase was observed in the present study, which is in accordance with the studies of Varnai et al. (2011), who showed that during hydrolysis of Avicel, steam-pretreated spruce and catalytically delignified spruce, the AnCel3A glucosidase was adsorbed to a lesser extent than *T. reesei* cellulases. This can be partially due to that AnCel3A has no CBM. Similar findings were also reported by Sipos et al. (2010) during hydrolysis of steam-pretreated spruce. In contrast, β-glucosidase has been reported to adsorb more strongly to lignin-rich residues than endoglucanases do (Rahikainen et al., 2011).

4. Conclusions

Comparisons of the protein adsorption of eight lignocellulosic substances revealed differences between the adsorption patterns of different varieties of enzymes. The adsorption affected by the different lignocellulosic substances was difficult to attribute to properties such as surface area and pore size, but differences in carboxylic acid content and temperature during binding gave more consistent effects pointing towards hydrophobic interactions playing an important role. Investigations in this area could help design processes that facilitate productive binding and have lower enzyme dosages. Studies of enzyme adsorption employing advanced quantitative proteomics appear as a promising alternative for further research in the field.

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