Improvement of Enzymatic Saccharification of Unbleached Cedar Pulp with Amphipathic Lignin Derivatives

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Synthesized amphipathic lignin derivatives comprised of acidic acid lignin (AL) with poly(ethylene glycol) diglycidyl ether (PEGDE), ethoxy-(2-hydroxy)-propoxy-poly(ethylene glycol) glycidyl ether (EPEG) or dodecyloxy-poly(ethylene glycol) glycidyl ether (DAEO) were added before the enzymatic saccharification of unbleached cedar pulp along with two commercially available cellulases, Meicelase and Genencor GC220. At the same filter paper unit (FPU) dosage, GC220 showed higher sugar yield than Meicelase. The difference was attributed to the composition of processive and non-processive endoglucanase activities per FPU; GC220 had higher such activities than Meicelase. The sugar yield was significantly improved by the addition of the lignin derivatives. In addition, residual activities after the saccharification were maintained at the higher level by their addition than with polyethylene glycol (PEG) 4000. In particular, EPEG-AL yielded the complete recovery of cellulase activity when using 20 FPU/g of substrate. It was found that the lignin derivatives were directly associated with Cel6A, one of cellulase components, whereas PEG 4000 was not. Thus, it is evident that the lignin derivatives are promising agents to improve the enzymatic saccharification of cellulase.

Keywords: Amphipathic lignin derivatives; Enzymatic saccharification; Recovery of cellulase activity.

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INTRODUCTION

Our dependence on fossil resources and concomitant release of greenhouse gases causing climate change have put an intense focus on exploitation of alternative renewable resources for the production of fuels and chemicals (Horn and Eijsink 2010). Ligno-cellulosic materials are considered to be one of the most promising renewable resources as an alternative (Alkasrawi *et al*. 2003). These materials are abundant, with an estimated annual worldwide production of 10 to 50 billion tons on dry weight (Alfaro *et al*. 2009). Japanese cedar (*Cryptomeria japonica*) is a major wood species accounting for approximately 60% of the softwood plantation in Japan (Baba *et al*. 2011). To effectively use the plantation wood, in particular timber from forest thinning, bioethanol production from low-quality wood is one of the major focuses of research in Japan. The Forestry and Forest Products Research Institute (FFPRI), Tsukuba, Japan, recently built a pilot plant in Akita prefecture for bioethanol production from cedar wood, utilizing enzymatic

saccharification with cellulase. In this pilot plant, soda pulping is used as the delignification process (Magara *et al.* 2010). A dominant obstacle for the saccharification process lies in the high price of the cellulases, which accounted for as much as 60% of the total process cost for bioethanol production in the 1980's (Deshpande *et al*. 1984a). More recently, the cost of cellulase still accounts for 25% to 50% of the total cost for ethanol production (Himmel *et al*. 1997).

Maintaining cellulolytic activity, along with the reuse of cellulase, is one of the most important factors for reducing the enzyme cost (Das *et al.* 2011). However, the repeated use of cellulase seems to be practically impossible, because the cellulase activity inevitably decreases during the enzymatic saccharification process. The main reason for the gradual decrease in cellulolytic activity is initially due to the irreversible adsorption of cellulase onto the substrate. When lignocellulose, which is composed of cellulose, hemicellulose, and lignin, is used as a substrate, the cellulase strongly adsorbs onto the substrate mainly due to two interactions (Eriksson *et al*. 2002; Li *et al*. 2012). One is a specific interaction based on the formation of enzyme-substrate complex. This interaction covers a reversible, productive adsorption of cellulase on the substrate for saccharification and an irreversible, non-productive adsorption that reduces residual cellulase activity (Kristensen *et al*. 2007; Ma *et al*. 2008; Jalak and Valjamae 2010). The other one is a non-specific interaction between cellulase and residual lignin in the substrate, which is also an irreversible, non-productive adsorption. The interaction is caused by hydrophobic interaction and hydrogen bonding (Kristensen *et al*. 2007; Borjesson *et al*. 2007; Xu *et al*. 2008). Due to these non-productive adsorptions, cellulase activity eventually is reduced during enzymatic hydrolysis. Therefore, suppression of these kinds of non-productive adsorption is one of the targets to make enzymatic saccharification more economically feasible.

Immobilization of cellulase is one of the possible concepts to enable its repeated use (Karube *et al*. 1977). However, when cellulase was immobilized onto a solid polymeric support, saccharification efficiency was decreased because of a solid-solid phase reaction between the immobilized cellulase and the lignocellulosic substrate (Wongkhalaung *et al*. 1985). To overcome this drawback, an alternative system was proposed: cellulase was immobilized onto a water-soluble polymeric support to improve efficiency of saccharification and recovery of cellulase (Woodward 1989). Our research group reported a more useful, water-soluble polymeric support in 2001; as such a polymer, an amphipathic lignin derivative was prepared by the reaction of lignin with poly(ethylene glycol) diglycidyl ether (PEGDE) (Uraki *et al*. 2001). When cellulase was used together with PEGDE-lignin in a saccharification system for unbleached pulp, the sugar yield was higher than without the PEGDE-lignin; in addition, 80% of the cellulase activity was recovered after the fifth recycle of the cellulase from enzymatic saccharification. In 2007, polyethylene glycol (PEG) with its molecular mass of more than 4000 (PEG 4000) was reported to improve the cellulolytic saccharification of steam-pretreated spruce: 50% of the substrate was hydrolyzed during saccharification process without PEG 4000, while 78% with PEG 4000 (Börjesson *et al*. 2007). In addition, PEG 4000 increased the residual filter paper activity: only 5% of filter paper activity was found to be maintained after saccharification process without PEG 4000, while 56% with PEG 4000 (Sipos *et al*. 2010). Another study, using wheat straw lignocelluloses as a substrate, also reports that the cellulose conversion was improved from 51% (without PEG 6000) to 65% (with PEG 6000) (Kristensen *et al*. 2007). The usefulness of PEG was reported to be attributable to prevention of the non-productive association between the

cellulase and the substrate caused by hydrogen bonding (Xu *et al.* 2008; Li *et al*. 2012). Similarly, Tween 80, a non-ionic surfactant, was also reported to facilitate hydrolytic enzyme recycling for pretreated lodgepole pine. The result showed that the addition of Tween 80 could save 60% of the total enzyme cost for the saccharification of ethanol-pretreated lodgepole pine (Tu and Saddler 2009). Thus, non-ionic surfactants are expected to be chemical adjuncts to reduce the hydrolysis cost of lignocellulosics.

Very recently, we have confirmed the effect of PEGDE-lignin on the saccharification of unbleached cedar pulp, which was produced as a partially delignified substrate in the aforementioned pilot plant (Bardant *et al*. 2010). We have further developed two other types of amphipathic lignin derivatives with a stronger surface activity than PEGDE-lignin; EPEG- and DAEO-lignin (Homma *et al*. 2008, 2010). The other types of lignin derivatives are expected to improve the efficiency of saccharification more significantly, although the mechanism of how amphipathic lignin derivatives work during the saccharification has not been determined yet. In this study, the influence of amphipathic lignin derivatives on the cellulolytic saccharification of unbleached cedar pulp was investigated and compared with the effect of PEG 4000. In addition, the interaction between amphipathic lignin derivatives and cellulase was also examined by using the Biacore™ system, an analytical instrument based on surface plasmon resonance (SPR), to clarify the function of the lignin derivatives on the saccharification.

EXPERIMENTAL

Unbleached Cedar Pulp as a Substrate for Saccharification

Unbleached cedar pulp was prepared by soda pulping of cedar chips (1 kg) with 260 g of NaOH in 6 L of water (Bardant *et al*. 2010). The time to cooking temperature was 90 min. (170 °C), and the time at cooking temperature was 150 min. The crude pulp was washed with 1% NaOH and water, successively, and filtered by pressing to a moisture content of 70%. The pulp was lyophilized to remove the residual moisture to yield a dry pulp. The pulp's lignin content was determined using the Klason method (TAPPI T-222 om-83), as insoluble residue through filtration. The filtrate was subjected to neutral sugar analysis after the neutralization with $Ba(OH)_2$ followed by removal of precipitate (Slavin and Marlett 1983). The determination of sugars was carried out on an HPLC device (Shimadzu LC10 system, Kyoto, Japan) equipped with a corona charged aerosol detector (ESA Biosciences Inc., Chelmsford, MA, USA). The column used was Shodex SUGAR SP0810 with SUGAR SP-G as a guard column (Showa Denko Co. Ltd., Tokyo, Japan), and the column temperature was 80 °C. The eluent used was Milli-Q purified water, and its flow rate was set at 0.5 mL/min. The injection volume was 20 μL.

Amphipathic Lignin Derivatives

Acetic acid lignin (AL) was prepared from the spent liquor of the atmospheric acetic acid pulping of birch wood (Uraki *et al*. 1991). Amphipathic lignin derivatives were prepared by the reaction of AL with poly(ethylene glycol) diglycidyl ether (PEGDE), ethoxy-(2-hydroxy)-propoxy-poly(ethylene glycol) glycidyl ether (EPEG), or dodecyloxy-poly(ethylene glycol) glycidyl ether (DAEO), as shown in Fig. 1, in aqueous 1 M NaOH solution according a previous report (Homma *et al*. 2010). EPEG was prepared according to a previous report (Homma *et al*. 2010). After the derivatization, the reaction solution was acidified to pH 4, and purified by ultrafiltration (cut-off molecular

mass, 1000 Da). Finally, amphipathic lignin derivatives were lyophilized to remove the remaining water. PEG contents of PEGDE-, EPEG-, and DAEO-AL were 61.2, 72.3, and 68.4%, respectively, and were determined by the modified Morgan method (Homma *et al*. 2008; Siggia *et al*. 1958; Morgan 1946).

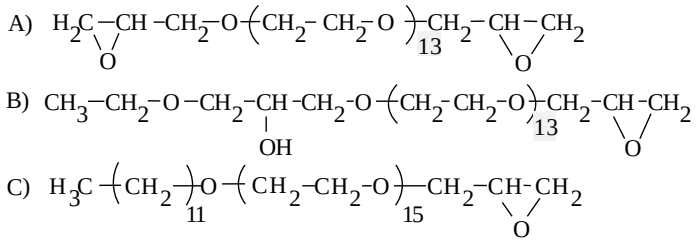

Fig. 1. Chemical structure of (A) poly(ethylene glycol) diglycidyl ether (PEGDE), (B) ethoxy-(2-hydroxy)-propoxy-poly(ethylene glycol) glycidyl ether (EPEG), and (C) dodecyloxy-poly(ethylene glycol) glycidyl ether (DAEO)

Enzymes and Measurement of Their Activities

Two types of commercially available cellulases, Meicelase (powder form; Meiji Seika Co. Ltd., Tokyo, Japan) and Genencor GC220 (liquid form; Genecor International Inc., USA; Lot # 4901121718), were used for the experimental saccharification. The cellulolytic activity expressed as filter paper units (FPUs) which was measured using the NREL technical report, NREL/TP-510-42628, method (Ghose 1987). The enzyme activity of Meicelase and Genencor GC220 as received were 0.24 FPU/mg and 66.1 FPU/mL, respectively.

β-Glucosidase activity of the cellulases was measured according to Berghem and Petterson (1974) method, using the substrate *p*-nitrophenyl-*β*-D-glucopyranoside (*p*NPG). Endoglucanase activity was measured using 2% carboxymethyl cellulose in 0.05 M sodium citrate buffer (pH 4.8) (Ghose *et al*. 1987). The activity of Cel5A (EG II) and Cel7A (CBH I) was measured using *p*-nitrophenyl- *β*-D-lactoside (*p*NPL) in accordance with Desphande *et al*. (1984b).

Saccharification of Unbleached Cedar Pulp

Each amphipathic lignin derivative (10% of substrate on dry weight basis), as well as PEG 4000 (purchased from Wako Pure Chemicals (Osaka, Japan)), was dissolved in 150 mL of 50 mM citrate buffer (pH 4.8). Cellulase (10 FPU/g, and 20 FPU/g of substrate) was added to the solution, and the mixture was stirred for 1 h. Finally, 1.5 g of unbleached pulp was added to the solution, and the suspension was gently shaken at 50 °C for 48 h. After saccharification, the suspension was filtered through a G4 glass filter. The precipitate was washed three times with the buffer solution, and weighed after complete drying at 105 °C. The sugar yield (SY) was calculated, using the following equation:

$$SY\ (\%) = (WS - WR) \times 100 / WS \quad (1)$$

where WR is the weight of residue (g) after saccharification, and WS is the initial weight of substrate (g).

The filtrate was subjected to ultrafiltration with a membrane filter (cut-off molecular mass 1000 Da). The composition and concentration of neutral sugars in the

filtrate were determined by using the HPLC method mentioned above. The residual enzyme solution (*ca*. 30 mL), as an unfiltered fraction concentrate, was diluted with 250 mL of the buffer solution, and subjected again to ultrafiltration up to 30 mL of unfiltered fraction. This process was repeated three times. Finally, the recovered enzyme solution was further ultrafiltered to the volume of 15 mL. Filter Paper Activity (FPA) of the concentrated enzyme solution was measured, and a recovered activity of cellulase (%) was calculated as a ratio of remaining activity to initial cellulase activity using the following equation:

$$\text{Recovered Activity (\%)} = \frac{\text{FPU after saccharification (unit/g) x 100}}{\text{FPU of initial cellulase (unit/g)}} \quad (2)$$

Interaction between Amphipathic Lignin Derivatives or PEG 4000, and CBH II (Cel6A)

CBH II (Cel6A) was isolated from Novozyme NS50013 in accordance to literature reports (Reinikainen *et al.* 1995; Samejima *et al*. 1997). The isolated enzyme was immobilized on a sensor chip CM-5 by amino coupling in a Biacore-X (GE, Healthcare, Japan). Briefly, the carboxyl group was converted to an active ester form with *N*-hydroxysuccinimide and 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride. The enzyme in 1 M citrate buffer solution (pH 4.5) was introduced into a flow cell (flow cell 1), which runs on the surface of the activated sensor chip. After washing with the buffer, ethanolamine hydrochloride solution was introduced into the flow cell to block the unreacted active ester. The other flow cell (flow cell 2) was also blocked with ethanolamine hydrochloride without enzyme immobilization. Finally, each of the amphipathic lignin derivatives and PEG 4000 in 50 mM citrate buffer solution were introduced to both flow cells up to a constant adsorption at a flow rate of 20 μL/min and 37 °C, and then only buffer solution was introduced for desorption. An adsorption-desorption isotherm was obtained by the difference in the adsorbed amount between the flow cell 1 and 2. A binding constant was calculated by using BIA evaluation software ver. 4.1 (GE, Healthcare, Japan).

The complete desorption of adsorbed lignin derivatives or PEG 4000 on sensor chip was carried out with an aqueous 0.1 wt.% Triton X-100 solution, and the regenerated sensor chip was used repeatedly for adsorption-desorption experiments.

The molecular mass was obtained with a SEC-MALLs device (Wyatt, Santa Barbara, CA, USA), with a refractive index detector and a light-scattering detector. Three columns of Shodex K-805L (Showa Denko Co. Ltd., Tokyo, Japan) were connected and used. The column temperature was 40 °C. The eluent was chloroform, and flow rate was 1.0 mL/min.

RESULTS AND DISCUSSION

Cellulolytic Saccharification of Unbleached Cedar Pulp

Three types of lignin derivatives (EPEG-AL, DAEO-AL, and PEGD-AL) and PEG 4000 were employed for the cellulolytic saccharification of unbleached cedar pulp using two types of commercially available cellulases. The unbleached cedar pulp had a

Klason lignin content of 6.75%, and a neutral sugar content of 75.1% for glucose and 6.9% for mannose.

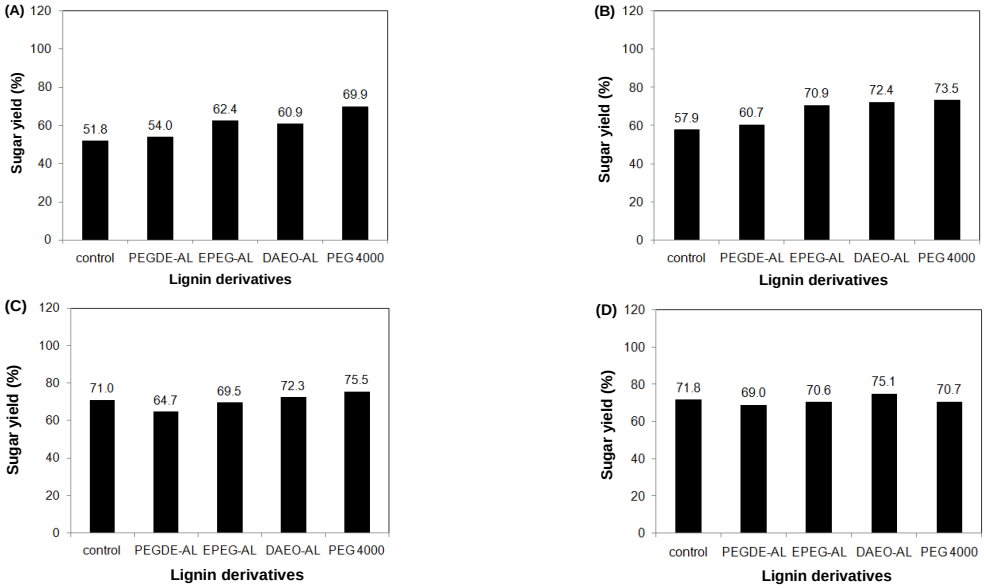

Fig. 2. Sugar yield of unbleached cedar pulp, using Meicelase (A) and Genencor GC220 (B) at the initial cellulase activity 10 FPU/g substrate; Meicelase (C) and Genencor GC220 (D) at the initial cellulase activity 20 FPU/g substrate at pH 4.8 and 50 °C.

Figure 2 (A and B) shows the sugar yield (SY) at the cellulase activity of 10 FPU/g of substrate. Comparing SYs of the two cellulases without additive, GC220 showed higher SY than the Meicelase. PEG 4000 dramatically improved the SYs of both cellulases, although the amphipathic lignin derivatives also improved SYs significantly. Interestingly, compared to the controls, PEGDE-AL apparently showed a negative effect on SY at 20 FPU/g, while PEGDE-AL gave a positive effect on SY at 10 FPU/g. This negative effect is further discussed in a later section. However, the higher positive effect of PEG 4000 was only observed for the SY of Meicelase at 20 FPU/g of substrate, as shown in Fig. 2 (C and D). In the case of GC220 at 20 FPU/g of substrate, DAEO-AL showed the highest SY of all the additives examined, and the other SYs were almost identical within the experimental error. In the filtrate, the only water-soluble product detected by HPLC from saccharification was glucose. The maximum yield of glucose obtained (75.1%), using DAEO-AL, was almost identical to the glucose content measured in the cedar pulp (75.1%). Thus, cellulose in the unbleached cedar pulp was almost quantitatively hydrolyzed.

The order of increasing surface activity was reported by Homma *et al*. (2010) as PEGDE-AL < EPEG-AL << DAEO-AL. Therefore, these results suggested that SY may be related to surface activity and that PEG 4000 was not necessarily the best additive to improve SY.

Recovery of Cellulase Activity after One-Time Saccharification of Cedar Pulp

Figure 3 (A-D) shows the recovery of cellulase activity after one-time saccharification at the initial activity of 10 and 20 FPU/g of substrate. Addition of all lignin derivatives (EPEG-AL, DAEO-AL, and PEGDE-AL) obviously improved the recovery of cellulase activity. In particular, in the case of GC220 at 20 FPU/g of substrate,

PEGDE-AL and EPEG-AL facilitated the complete recovery of cellulase activity (Fig. 3D). In the case of Meicelase at 20 FPU/g of substrate, however, PEGDE-AL and EPEG-AL did not show 100% recovery, though they improved recovery of cellulose activity relative to the control. This difference in recovery of cellulase activity between Meicelase and GC220 is discussed in a later section.

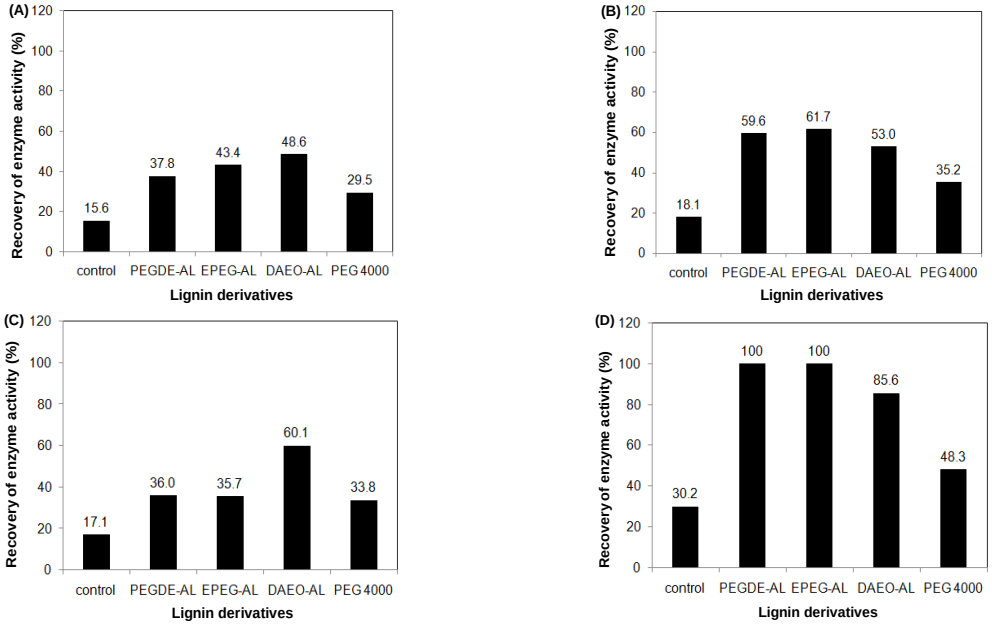

Fig. 3. Recovery of cellulose activity of unbleached cedar pulp, using Meicelase (A) and Genencor GC220 (B) at the initial cellulase activity 10 FPU/g substrate; Meicelase (C) and Genencor GC220 (D) at the initial cellulase activity 20 FPU/g substrate at pH 4.8 and 50 °C

On the other hand, PEG 4000 also had a positive effect on the recovery of cellulase activity; however, the increment with PEG 4000 was much smaller than with the lignin derivatives. High recovery of cellulase activity by lignin derivatives is expected to enable repeated use of the cellulase, although it is necessary to separate it from the glucose in the saccharification medium. In addition, this result suggests that it would also be possible to charge the lignocellulosic substrate successively into saccharification system with little or no additional charge of cellulase.

Interaction of Amphipathic Lignin Derivatives and PEG 4000 with CBH II

The cellobiohydrolases, Cel6A and Cel7A, which have both catalytic domain and cellulose-binding domain, are major cellulase components secreted from *Trichoderma reesei* (Palonen *et al*. 1999; Watson *et al*. 2009; Igarashi *et al*. 2011). To investigate the direct interaction between the cellobiohydrolases and the additives to improve saccharification of lignocellulose, we successfully isolated pure Cel6A from a commercial cellulase, and immobilized it on a sensor chip to be studied by surface plasmon resonance (SPR). The immobilized amount of the enzyme was 2024 RU (1 RU = 1 pg/mm^2). Each citrate buffer solution containing amphipathic lignin derivatives, and PEG 4000 as analytes was applied to the resultant sensor chip to monitor their adsorption and desorption behaviors.

Figure 4 shows the sensorgrams of the adsorption and desorption processes of the analytes. A separate experiment indicated that DAEO-AL adsorbed onto Cel6A much

more significantly than the other amphipathic lignin derivatives when measured at the same concentration, which made accurate adsorption-desorption measurements impossible. Thus, the adsorption-desorption behavior of DAEO-AL was measured at one-hundredth the concentration range (0.001 to 0.008 g/L) when compared to those for PEGDE-AL and EPEG-AL (0.1-0.8 g/L).

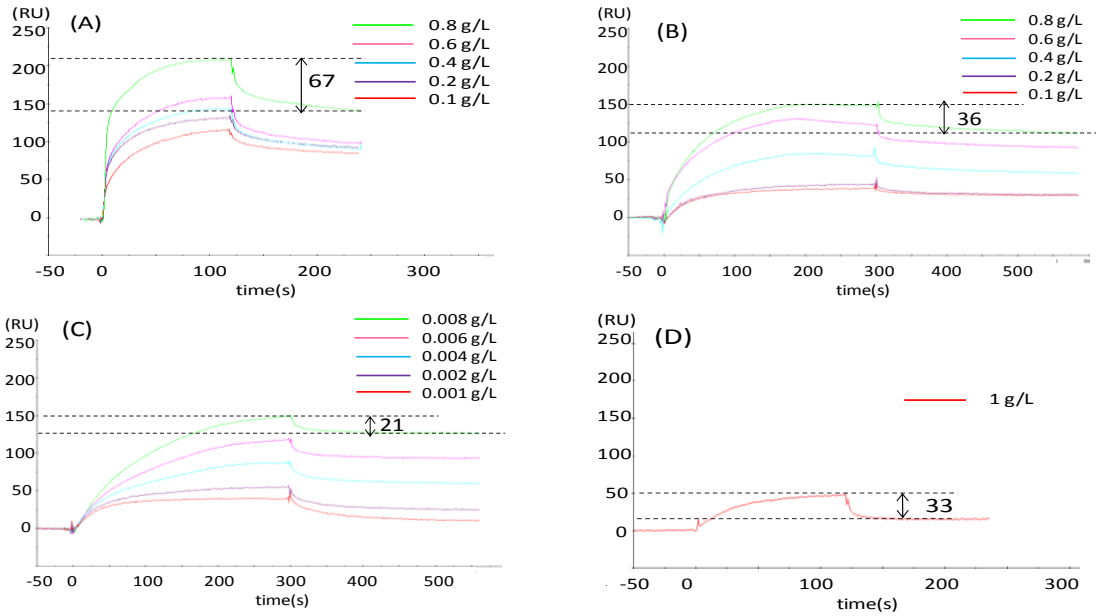

Fig. 4. Sensorgram showing the interaction between Cel6A immobilized on a CM5 sensor chip and the amphipathic lignin derivatives as analytes: (A) PEGDE-AL; (B) EPEG-AL; (C) DAEO-AL; and (D) PEG 4000. The arrows and values in the figures indicated desorbed amount at the highest concentration of analytes.

The amount of desorption of DAEO-AL, EPEG-AL, and PEGDE-AL was 14%, 24%, and 32% of the amount of the adsorption, respectively. It is assumed from these results that DAEO- and EPEG-AL have a higher affinity to Cel6A than PEGDE-AL. On the other hand, PEG 4000 showed very little adsorption even at the highest order of concentration (1 g/L). Furthermore, 66% of the PEG 4000 adsorbate was released. This result suggested that PEG 4000 does not directly interact with the active site(s) of the cellulase enzyme.

In order to compare the binding affinity of the lignin derivatives with cellulase, we calculated a binding constant of the lignin derivatives to Cel6A on the basis of the following assumptions. First, each lignin derivative binds with cellulase in such a manner as one molecule-to-one molecule (one-on-one) interaction. Second, two types of weight-average molecular mass (Mw) for each lignin derivative obtained by SEC-MALLS were used for the calculation; one was obtained by refractive-index detector (RI), while the other by light-scattering detector (LS). The latter value may be overestimated, since this light scattering detector did not have a filter to eliminate self-fluorescence of lignin. The calculated results are shown in Table 1. The big difference in Mw between RI and LS might be attributed to lignin conformations in the solution. Isolated lignins showed a very small exponential parameter in Mark-Houwink-Sakurada equation (Goring 1971). In addition, an amphipathic polymeric molecule has a dense structure upon self-aggregation. Therefore, RI showed smaller values. EPEG- and DAEO-AL have four to six digits greater binding constants (Ka-RI and Ka-LS) than PEGDE-AL, as anticipated. The

binding constant, Ka, might be related to surface activity. The high affinity affected the sugar yield and recovery of enzyme activity.

Table 1. Weight-Average Molecular Mass of the Amphipathic Lignin Derivatives Measured by SEC-MALLS and Binding Constant Calculated on the Basis of One Molecule-to-One Molecule Interaction

Lignin Derivatives	RI (Mw)	K_a-RI (1/M)	LS (Mw)	K_a-LS (1/M)
PEGDE-AL	1260	4.01×10^6	2.05×10^5	1.74×10^4
EPEG-AL	1460	8.38×10^{10}	7.79×10^6	1.05×10^9
DAEO-AL	1900	1.17×10^{12}	1.59×10^6	7.04×10^9

RI, Weight-average molecular mass was obtained using a refractive-index detector. LS, Weight-average molecular mass was obtained using a light-scattering detector. K_a-RI and K_a-LS are binding constants calculated on the basis of Mw obtained by RI and LS, respectively.

Mechanism on the Improvement Effect of Amphipathic Lignin Derivatives on the Cellulolytic Saccharification

Cellulase consists of three major components, cellobiohydrolase, endoglucanase, and *β*-glucosidase (Becham *et al*. 2012; Jalak *et al*. 2012). Endoglucanases (EG; Cel5A and Cel7B) and cellobiohydrolases (CBH; Cel6A and Cel7A) are the main cellulase components to depolymerize cellulose chain. On the other hand, *β*-glucosidase cleaves oligosaccharides to yield glucose and does not have a cellulose-binding domain (CBD), which points to a low affinity towards cellulose as a solid substrate (Teeri 1997; Palonen *et al*. 2004).

Table 2. Hydrolytic Activities of Commercially Available Cellulases

Substrate	Meicelase (unit/FPU)	Genencor GC220 (unit/FPU)
CMC	67.0	163
*p*NP-lactoside	1.43	3.50
*p*NP-glucoside	8.20	2.65

CMC, Carboxymethyl cellulose is a substrate for endoglucanase activity measurement; *p*NP-lactoside, *p*-nitrophenyl-*β*-D-Lactoside is a substrate for Cel5A and Cel7A activity measurement; *p*NP-glucoside, *p*-nitrophenyl-*β*-D-Glucopyranoside is a substrate for *β*-Glucosidase activity measurement. These enzyme activities (unit) were expressed on the amount of cellulase that gives 1 FPU.

Genencor GC220 consists of larger amounts of EG and CBH (163 unit/FPU for CMC and 3.5 unit/FPU for *p*NP-lactoside) than Meicelase, whereas Meicelase is rich in *β*-glucosidase (Table 2). Table 3 shows all lignin derivatives had a positive effect on FPU activity of Genencor GC220, but a negative effect on that of Meicelase except for EPEG-AL. These effects of the lignin derivatives on the enzyme activity suggested that the lignin derivatives activated endoglucanase activity upon the direct association, as mentioned above, and suppressed *β*-glucosidase activity. The difference in the action of lignin derivatives to the cellulases would affect the recovery of the FPU activity after saccharification.

FPU is defined as the amount of enzyme required to liberate 1 μmol of glucose from cellulose per min. The generated glucose is determined for initial 1-h enzymatic hydrolysis in FPU measurement. This value is not an average amount of glucose production per min for 24-h or 48-h enzymatic saccharification Therefore, an initial production rate of glucose affects FPU value. In the FPU measurement of Meicelase, production of cellobiose and related oligosaccharides must be a rate-limiting step because the glucose

conversion quickly proceeds due to the fact that Meicelase is rich in *β*-glucosidase; endoglucanase is the key enzyme. On the other hand, *β*-glucosidase activity dramatically influenced the FPU measurement of GC220, because GC220 had only one-third the *β*-glucosidase activity of Meicelase.

Table 3. FPU Activity of Commercially Available Cellulases in the Presence of Additives

Additives	Meicelase activity (FPU/mg of enzyme)[a)]	Genencor GC220 activity (FPU/ml of enzyme)
PEGDE-AL	0.189	96.9
EPEG-AL	0.238	85.1
DAEO-AL	0.198	76.7
control	0.211	66.1

[a)] This activity measurement was conducted after one year storage in refrigerator at 4°C since it was received. Thereby, the control activity was slightly reduced than initial activity as received (0.24 FPU/mg of enzyme).

In the saccharification at the initial cellulase activity of 10 FPU/g substrate, the activities of both commercial enzymes were insufficient to achieve complete saccharification (Fig. 2 A and B). Therefore, it was anticipated that cellulase adsorption onto substrate resulted in the reduction of residual FPU activity. In particular, endoglucanase adsorption in the saccharification with Meicelase would remarkably affect the reduction of residual FPU activity. In fact, the recovery of Meicelase (Fig. 3A), having smaller endoglucanase activity than GC220 (Table 2), was smaller than that of GC220 (Fig. 3B).

At 20 FPU/g, PEGDE- and EPEG-AL apparently showed the complete recovery of the GC220 activity (Fig. 3D). If the same amount of endoglucanase in Meicelase and GC220 adsorbed on the substrate, a significant amount of the residual endoglucanase still remains in GC220, which can supply sufficient amount of cellobiose as a substrate to *β*-glucosidase. Thus, no reduction of residual FPU activity in GC220 was observed under such conditions. On the other hand, the residual endoglucanase activity in Meicelase must be very small. Therefore, apparent reduction of FPU in Meicelase was clearly observed, even by the addition of such amphipathic lignin derivatives at the initial cellulase activity of 20 FPU/g substrate.

Finally, we discuss the lower SY in the presence of PEGDE-AL at 20 FPU/g than that of control in Fig. 2 C and D. PEGDE, carrying two glycidyl groups, bridges between lignin macromolecules. PEGDE-AL, then, has a hydrophobic domain more frequently than EPEG- and DAEO-ALs. The hydrophobic domain of PEGDE-AL may sometimes act as an inhibitor of cellulase, covering the active sites of the enzyme and preventing the substrate from insertion into the active sites. But it should be noted that interaction between PEGDE-AL and Cel6A is very weak (Table 1), and thus PEGDE-AL can promote the recovery of cellulase activity (Fig. 3D).

CONCLUSIONS

1. Amphipathic lignin derivatives were found to improve the sugar yield of lignocellulose saccharification, as well as PEG 4000.

2. The amphipathic lignin derivatives dramatically improved the recovery of cellulase activity after one-time saccharification as compared with PEG 4000. This effect was more significant for Genencor GC220 containing EG and CBH activities than Meicelase. These results suggested that the lignin derivatives make it possible to use cellulase repeatedly.

3. The difference in the mechanism for improved saccharification between the lignin derivatives versus PEG 4000 was attributed to the interaction with processive endoglucanase using surface plasmon resonance (SPR); the amphipathic lignin derivatives directly interacted with the enzyme, whereas PEG 4000 did not.

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