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Title: Association of amphipathic lignin derivatives with cellobiohydrolase groups improves enzymatic saccharification of lignocellulosics

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Abstract

Amphipathic lignin derivatives (ALDs), prepared from hardwood acetic acid lignin and softwood soda lignin via coupling with a mono-epoxylated polyethylene glycol, have been reported to improve the enzymatic saccharification efficiency of lignocellulose while maintaining significant residual cellulase activity after saccharification. We previously demonstrated that the effect of ALDs was caused by a direct interaction between

ALDs and Cel6A (or CBH II). In this study, a different ALD was prepared from softwood kraft lignin in addition to aforementioned ALDs. The interactions between all the ALDs and the enzymes other than Cel6A, such as Cel7A and Cel7B, in a cellulase cocktail were investigated using surface plasmon resonance. The kraft lignin-based ALD showed the highest residual cellulase activity among all ALDs and an improved cellulolytic enzyme efficiency similar to those of the other ALDs. All ALDs were found to directly associate with major enzymes in the cellulase cocktail, Cel6A and Cel7A (or CBH I), but not with Cel7B (or EG I). In addition, the ALDs showed a much higher affinity to amino groups than to hydroxy and carboxy groups. In contrast, polyethylene glycol (molecular mass 4000 Da), one part of the ALD and a previously reported enzymatic saccharification enhancer, did not adsorb onto any enzymes in the cellulase cocktail or the amino group. Size exclusion chromatography demonstrated that the ALDs formed self-aggregates in both water and chloroform; the formation process in the latter was especially unique. Therefore, we conclude that the high residual cellulase activity is attributed to the direct association of ALD aggregates with the CBH group.

Keywords: Amphipathic lignin derivative, Aggregation, Cellulase, Enzymatic saccharification, Surface plasmon resonance

1. Introduction

Lignocellulosic biomass is a promising alternative to fossil resources due to its abundance on earth, renewability, carbon neutral property, and lack of competition with food production (Valentine et al. 2012). The production of bioethanol, as a liquid fuel as well as a platform compound for value-added chemicals, from polysaccharide components in biomass has drawn much attention (Kamm and Kamm 2004). The production process consists of saccharification, fermentation and distillation. Among the two proposed saccharification methods, acidic and enzymatic saccharification, the latter is more promising because the reaction conditions are mild and no special reaction vessels are required, as corrosion is not an issue in enzymatic reactions (Sun and Cheng 2002). However, enzymatic saccharification also has disadvantages compared to acid saccharification, such as the high preparation cost of cellulolytic enzymes (cellulases) and low catalyst recovery (Deshpandet and Eriksson 1984). The low cellulase recovery is caused by the non-productive interaction between cellulase and cellulose and the non-specific hydrophobic interaction between cellulase and lignin in lignocelluloses (Eriksson et al. 2002). Through these

50 interactions, the activity of cellulase is markedly decreased during enzymatic saccharification, and the reuse of
51 cellulase is very difficult.
52 Non-ionic surfactant additives, such as Tween 20, Tween 80 and Triton X-100, improve saccharification
53 efficiency by inhibiting and/or suppressing undesired macromolecular interactions (Park et al. 1992; Eriksson et al.
54 2002; Seo et al. 2011). Polyethylene glycol (PEG) with a molecular mass greater than 4000 Da, a constituent moiety
55 of Tween 20, Tween 80 and Triton X, also improves saccharification efficiency (Börjesson et al. 2007a, b). Lignin
56 analogues, such as lignosulfonate (Nakagame et al., 2011; Lou et al. 2013; Zhou et al. 2013; Wang et al. 2013),
57 organosolv lignins (Lai et al. 2014) and water-soluble lignin derivatives (Uraki et al. 2001; Lin et al. 2015a, b), have
58 been reported to achieve similar performance improvements. Improvement mechanisms to suppress cellulase-
59 binding interactions may be classified into two groups: interactions between the additive and the lignocellulosic
60 substrate and interactions between the additives and the cellulase itself. Most non-ionic surfactants, including PEG,
61 belong to the former group (Eriksson et al. 2002; Börjesson et al. 2007a, b; Kristensen et al. 2007). Although some
62 lignin analogues are classified into the latter group (Wang et al. 2013; Lou et al. 2014; Lin et al. 2015b), the
63 respective mechanism for cellulase activity improvement has not been elucidated.
64 Our research group has prepared amphipathic lignin derivatives (ALDs) through the reaction of isolated
65 lignins, such as hardwood acetic acid lignin (AL) and softwood soda lignin (SL), with PEG diglycidyl ethers (Uraki
66 et al. 2001, Lin et al. 2015a, b). We predicted that these lignins would act as water-soluble supports for the
67 immobilization of cellulase, to suppress non-productive interactions (Woodward 1989). In fact, the ALDs preserved
68 cellulase activity at a high level, long after the termination of saccharification, along with enhanced saccharification
69 efficiency (Uraki et al. 2001, Winarni et al. 2013). These ALD-associated improvements enable repeated use of
70 cellulase for lignocellulosic saccharification (Uraki et al. 2001, Winarni et al. 2014) and also contribute to improved
71 bioethanol production efficiency in simultaneous fed-batch saccharification and fermentation (Cheng et al. 2014).
72 The effect of ALDs on enzymatic saccharification was proposed to be due to the direct interaction of the ALDs and
73 enzymes in the cellulase cocktail, specifically the interaction between CBH II and ALDs, which was monitored
74 using surface plasmon resonance (SPR). We have not yet investigated the interactions of ALDs with other enzymes
75 in cellulases, but cellulases secreted from *Trichoderma reesei* include the cellobiohydrolases CBH I (current name
76 based on the family, Cel7A) and CBH II (Cel6A), endoglucanases EG I (Cel7B) and EG II (Cel5A), and *β*-

77 glucosidase (Cel3A) (Teeri and Henriksson 2009). Therefore, an investigation of the interactions between these
78 cellulase enzymes and the ALDs is one of the objectives of this study.
79 Recently, Lou and colleagues developed lignin-based polyoxyethylene ether as an additive to improve
80 saccharification efficiency (Lin et al. 2015a, b). The structures of these additives are nearly identical to those of our
81 ALDs. They demonstrated that the improved saccharification was caused by a direct interaction between the lignin
82 derivative and cellulase using a quartz crystal microbalance with dissipation monitoring (QCM-D). However, these
83 QCM-D investigations were carried out using a cellulase cocktail, not pure enzymes.
84 In this study, we purified CBH I, EG I and CBH II from secretions of *Trichoderma reesei* and investigated the
85 interactions between the ALDs and the purified enzymes using SPR. In addition, a new ALD was prepared from
86 softwood kraft lignin (KL) and subjected to enzymatic saccharification and interaction analysis. KL is easily
87 obtained or prepared worldwide from conventional kraft pulping mills, while AL and SL are produced in fewer mills
88 and regions.

89

90 **2. Experimental section**

91 2.1. Preparation of amphipathic lignin derivatives.
92 ALDs were prepared from AL (Uraki et al. 1991), SL (Cheng et al. 2014), and softwood KL (Aso et al. 2013)
93 with mono-epoxylated PEG, ethoxy-(2-hydroxy)-propoxy-polyethylene glycol glycidyl ether (EPEG: 48, 49-epoxy-
94 3,7,10,13,16, 19, 22, 25, 28, 31, 34, 37, 40, 43, 46-pentadecaoxanonatetracontane-5-ol in IUPAC nomenclature)
95 (Homma et al. 2008), the chemical structure of which is shown in **Figure 1**. AL, SL, and KL were separately
96 dissolved in 1 M NaOH aqueous solution and stirred for 24 h. EPEG was added to the solution and stirred for 2 h at
97 70 °C. The reaction was stopped by the addition of glacial acetic acid to pH 4. The reaction solution was purified by
98 ultrafiltration with a membrane filter (molecular mass cut-off of 1000 Da) (Advantec, Tokyo, Japan). The solid
99 residue was lyophilized to give the ALDs (EPEG-AL, EPEG-KL, and EPEG-SL). The details of these reactions
100 have been previously described (Homma et al. 2008, 2010).
101

102 2.2. Characterization of amphipathic lignin derivatives.

Size exclusion chromatography (SEC) was performed with a UV detector (SPD-M10A VP, Shimadzu Co., Kyoto, Japan), monitoring at 280 nm, and a multi-angle laser light scattering (MALS) detector, equipped with an option laser at 785 nm and a 785-nm inference filter kit (Wyatt Technology Co., CA, U.S.A.), using chloroform and water separately as eluents. Two Shodex K-803L linear columns (exclusion limit: 7.0×10^4, Showa Denko Co. Ltd., Tokyo, Japan) in tandem were used with the chloroform eluent. A Shodex GF-7M HQ column (exclusion limit: 1.0×10^7, Showa Denko Co. Ltd., Tokyo, Japan) was used with the water eluent. The column oven (CTO-10AC vp, Shimadzu Co., Kyoto, Japan) was maintained at 40 °C with a flow rate of 0.5 mL/min. Polystyrene (PS) standards with a molecular mass of 6.5×10^2 to 4.23×10^6 Da (Agilent Technologies, Santa Clara, CA, U.S.A.) and toluene were used to calibrate the Shodex K-803L columns. Polyethylene glycol standards with a molecular mass of 1.0×10^3 to 3.8×10^6 Da (Tosoh Co. Ltd. Tokyo, Japan) were used to calibrate the Shodex GF-7M HQ column. The weight-average molecular mass (Mw) was estimated from MALS data using the MALS software ASTRA 6.1.2, where dn/dc was measured on an automatic refractometer, Anton Paar Abbemat 550 (Graz, Steiermark, Austria).

Elemental analyses (carbon, hydrogen, nitrogen, and sulfur) of the isolated lignins and their derivatives were performed at the Instrumental Analysis Division, Global Facility Center, Creative Research Institution, Hokkaido University. The PEG content of the lignin derivatives was determined by Morgan's method (Morgan 1946; Siggia et al. 1958).

2.3. Enzymatic saccharification.

EPEG-AL, EPEG-SL, EPEG-KL, Tween 80, and PEG 4000 (0.1 g, 10% with respect to dried substrate) were separately dissolved in 100 mL of 50 mM citrate buffer (pH 4.8). A commercial cellulase, Meicelase (Meiji Seika Pharma, Tokyo, Japan), with 10 filter paper unit (FPU)/g pulp was added to the solution, which was then stirred for 1 h at room temperature. Dried unbleached softwood kraft pulp (1 g), which was kindly supplied by Nippon Paper Industries Co., Ltd. (Tokyo, Japan) and used as received, was added, and the mixture was incubated at 50 °C for 48 h with continuous shaking at 120 rpm. After saccharification, the reaction suspension was filtered through a 1GP16 glass filter with a pore size of 10-16 μm (Sibata Scientific Technology Ltd., Soka, Japan). The filtered residue was washed three times with 300 mL of 50 mM citrate buffer (pH 4.8) and dried at 105 °C. The sugar yield was calculated according to the following equation:

$$\text{Sugar yield (\%)} = (WS - WR)/WS \times 100$$

where WS (g) is the initial weight of the substrate and WR (g) is the weight of the filtered residue.

The filtrate was subjected to centrifugal ultrafiltration (VIVASPIN 20 with a 10-kDa cut-off membrane, Sartorius Co., Göttingen, Germany), and the residue on the membrane was washed with 45 mL of 50 mM citrate buffer (pH 4.8) three times using centrifugal ultrafiltration to remove small saccharides generated by the enzymatic saccharification. The recovered enzyme solution was subjected to a filter paper assay, according to the National Renewable Energy Laboratory (NREL) technical report (Adney and Baker, 2008). The residual cellulase activity of the recovered enzyme was calculated by the equation below.

Recovered activity (%) = (FPU after saccharification/FPU before saccharification) × 100

The FPU before saccharification was measured in the absence of the ALDs. The FPU after saccharification was measured in the presence of the ALDs when this saccharification was carried out with ALDs.

All of the measurements were conducted in duplicate, and the average values are reported.

2.4. Adsorption/desorption measurements of ALDs on enzymes using Biacore

2.4.1. Purification of cellulase components

A commercial cellulase, Celluclast 1.5 L (Novozymes Japan Co. Ltd., Chiba, Japan), secreted from *Trichoderma reesei*, was washed three times with 20 mM Tris-HCl buffer (pH 7.3) to replace the solvent via ultrafiltration with a polysulfone membrane (molecular mass cut-off of 10 kDa) (Toyo Roshi Kaisha Ltd., Tokyo, Japan).

CBH I was collected with a DEAE-TOYOPEARL 650S anion exchange column (Tosoh Co., Tokyo, Japan), using a linear gradient of 0.0 to 0.5 M NaCl in 20 mM Tris-HCl buffer (pH 7.3). The CBH I-rich fractions were further purified with a Phenyl-TOYOPEARL 650S hydrophobic column (Tosoh Co., Tokyo, Japan) using a linear gradient of 0.0 to 1.0 M $(NH_4)_2SO_4$ in 20 mM potassium phosphate buffer (pH 7.0). Similarly, EG I was purified from the residue after CBH I and II, according to the literature (Egusa et al. 2010). All procedures were conducted at 4 °C with a flow rate of 1.5 mL/min. Protein concentrations and 4-nitrophenyl β-D-lactopyranoside (*p*-NPL) activity were monitored to confirm the existence of CBH I by the Bradford protein assay (Bradford, 1976) and *p*-NPL activity measurements (Deshpande et al. 1984), respectively. Enzyme purity was confirmed by SDS-PAGE.

158 2.4.2. Preparation of sensor chips.

159 2.4.2.1. Conversion of carboxy groups on sensor chips to other hydrophilic functional groups

160 The carboxy groups on a CM-5 sensor chip (Biacore) were converted to amino and hydroxy groups as shown
161 in **Figure 2**. In both conversions, the carboxy group was first converted to an activated ester group via a reaction
162 with 0.4 M 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) followed by treatment with 0.1
163 M *N*-hydroxysuccinimide (NHS). The activated ester group was treated with 15 L of 40 mM cystamine chloride in
164 0.15 M sodium borate buffer (pH 5.8) to yield an amino group surface on the sensor chip. Similarly, the active ester
165 was reacted with ethanolamine to give a hydroxy group surface. The reactions were conducted in a Biacore-X flow
166 system with 10 mM HBS-EP [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, pH 7.4; GE Healthcare Japan,
167 Tokyo, Japan] running buffer solution at a flow rate of 5 L/min at 25 °C.
168

169 2.4.2.2. Immobilization of purified CBH I on a sensor chip

170 A scheme for the immobilization of CBH I on the sensor chip as well as the immobilization of the other
171 cellulose components, CBH II and EG I, is shown in **Figure 1**. CBH I was immobilized on the sensor chip by the
172 surface thiol coupling method (Sensor Surface Handbook 2008). An aqueous solution of 2-(2-pyridinyl-dithio)-
173 ethylamine (PDEA) (0.25 mL; 15 mg/mL) was added to 0.5 mg of CBH I in 6.6 mL of 0.1 M 2-(4-morpholino)
174 ethanesulfonic acid (MES) buffered solution, followed by 25 L of 0.4 M EDC. After 10 min, the mixture was
175 subjected to centrifugal ultrafiltration with VIVASPIN500 (Sartorius, Göttingen, Land Niedersachsen, Germany) at
176 3000 rpm for 15 min and then washed with 10 mM acetate buffered solution at pH 4.0 to exchange buffer solutions.
177 A sensor chip bearing cystamine was reduced with dithiothreitol to afford free thiols. The CBH I-bearing PDEA was
178 introduced to the sensor chip, blocking the thiol group with PDEA to give a CBH I-immobilized sensor chip. A
179 procedure based on the thiol-disulfide exchange reaction was carried out using a thiol coupling kit and a Biacore-X
180 flow system (GE Healthcare Japan Co., Tokyo, Japan) (Sensor Surface Handbook.2008). The reactions were
181 performed in 0.1 M MES running buffer (pH 5.0) at a flow rate of 5 μm/min at 25 °C.
182

183 2.4.2.3. Immobilization of purified CBH II and EG I on a sensor chip

As shown in **Figure 2**, CBH II and EG I were immobilized on a CM5 sensor chip via amine coupling using an amine coupling kit, following a previously described protocol (Sensor Surface Handbook 2008). In brief, CBH II and EG I in 10 mM acetate buffer solution at pH 4.0 were separately introduced to activated ester-bound sensor chips (compound **2** in **Figure 2**). Any unreacted activated esters remaining on the sensor chip were deactivated by the addition of ethanolamine. The reactions were performed in the Biacore-X flow system with 10 mM HBS-EP running buffer solution (pH 7.4) at a flow rate of 5 L/min at 25 °C.

2.4.3. Monitoring of interactions.

The interactions of the ALD and PEG 4000 analytes with the cellulase ligands immobilized on the sensor chip were monitored using a Biacore-X system. The ALD and PEG 4000 analytes were dissolved in 50 mM citrate running buffer (pH 4.8), and the analyte solutions were injected to a Biacore-X flow system. After each analyte was injected, only the running buffer was introduced to the measurement cell to monitor the desorption process. The monitoring was performed at a flow rate of 20 L/min at 37 °C with the citrate running buffer. After monitoring, the sensor chip was washed with 0.1 % (w/w) Triton X-100 aqueous solution to remove tightly bound analyte and to regenerate the ligand.

The interactions of several analytes with hydrophilic functional groups on the sensor chips were monitored with the citrate running buffer. The adsorption of analytes was calculated by subtracting the response value of the running buffer from the response value of the analyte.

Sensorgrams of the interactions between CBH I and the analytes were obtained by subtracting the adsorption of analytes on the amino group surface from the adsorption of analytes on CBH I, while the sensorgrams of CBH II and EG I with the analytes were obtained by subtracting the adsorption of analytes on the hydroxy group surface from the adsorption of analytes on CBH II and EG I, respectively.

3. Results and discussion

3.1 Characterization of ALDs

ALDs were prepared from three types of isolated lignins, AL, SL and KL, in which the aliphatic/phenolic hydroxy contents were 1.7/2.8, 5.5/4.4, and 3.1/4.1 mmol/g, respectively, as determined by the reaction of the

211 hydroxy group in the lignins with the epoxy group of EPEG. The elemental composition and PEG content of the
212 ALDs are shown in Table 1. As expected, only EPEG-KL contained sulfur, which was introduced by the pulping
213 process. EPEG-AL showed the highest PEG content among the ALDs, despite its low hydroxy content. This trend
214 may imply that the ring-opening addition of epoxylated PEG proceeds in a manner similar to graft polymerization: a
215 new hydroxy group is generated by the reaction of the hydroxy group in lignin with the epoxide, which then acts as
216 another reaction site.

217 To elucidate the interactions between the ALDs and enzymes in a cellulase cocktail, it is necessary to further
218 clarify the solution behavior of the ALDs as well as their molecular mass. A SEC-MALS analysis was conducted in
219 water and chloroform to obtain this information. For the MALS measurement, a new type of laser with a wavelength
220 of 785 nm was used, which is longer than the conventional wavelength of 658 nm. In addition, an interference filter
221 was used to avoid lignin self-fluorescence.

222 ALD self-aggregates were observed in water even at a low concentration of 0.5 g/L, and a unique aggregation
223 process was observed in chloroform. These aggregations in water and chloroform reflect the amphipathic property
224 of ALDs. The aggregation behavior in chloroform was examined first, followed by that in water, to elucidate the
225 ALD aggregation in relation to the aggregate mass.

226 **Figure 3A (a-d)** shows size exclusion chromatograms of EPEG-SL in chloroform monitored by a UV
227 detector at 280 nm. An intense peak with a relative molecular mass (upper X axis) of approximately 3 kDa was
228 observed, as shown in **Figure 3A-(a)**. The intensity of this peak decreased with increasing EPEG-SL concentration.
229 Concurrently, the intensity of the broad peak in the large mass fraction increased. The same tendency was also
230 observed for chromatograms of other ALDs, as shown in the **Supplementary material**. These results suggest that
231 all ALDs self-aggregate in chloroform, and the size of the aggregations can reach up to 2.0 g/L. The sharp peak at
232 low molecular mass was assigned to a single molecule of ALD. At the high ALD concentration of 3 mg/mL [**Figure**
233 **3A-(d)**], the broad peak at high molecular mass shifted to a smaller molecular fraction. This molecular size shift
234 suggests that the large aggregates comprise many loosely bound ALD molecules; at higher concentrations, these
235 large aggregates were converted to smaller, tighter aggregates, such as micelles or microcapsules.

236 **Figure 3-B** shows a chromatogram of EPEG-SL at 3.0 g/L obtained with a MALS detector (solid line). The
237 figure also contains calibration curves of absolute Mw obtained by MALS measurements of the specimen (dotted
238 line) and of relative molecular mass based on PS standards (dashed line). The dotted line is located above the dashed

239 line, suggesting that the EPEG-SL in chloroform exhibits a larger molecular mass (right; Y axis) than PS at the same
240 hydrodynamic radius or the same retention time. In other words, the ALD molecules became significantly more
241 compressed in the solvent compared to polystyrene. This behavior of ALD in organic solvent is consistent with
242 previous studies on the viscosity of isolated lignins. These compounds typically exhibit very low intrinsic viscosity
243 and low exponential values of α (0.15 to 0.32) (Goring 1971, Glasser et al. 1993; Oliveira and Glasser 1994; Dong
244 and Fricke 1995) in the Mark-Houwink-Sakurada equation, $[\eta] = KM^{\alpha}$. The value of α for PS in THF is 0.7. The
245 EPEG-SL results suggest that this ALD also has a compact conformation in chloroform, even after derivatization
246 with EPEG.

247 The absolute Mw of single EPEG-AL molecules in **Figure 3A-a** was estimated to be 2.26 x 10^4 g/mol by
248 extrapolating the linear region at 26-38 min in the dotted line to the peak at 41.4 min, as the Mw could not be
249 directly measured due to weak light scattering. Similarly, the absolute Mw values of EPEG-AL at 44.7 min and
250 EPEG-KL at 45.4 min were estimated and are summarized in Table 1.

251 Size exclusion chromatograms of all ALDs in water using a UV detector at 280 nm are shown in the
252 **Supplementary material**. All major peaks in the figures appear at the void volume of the column (1.0 x 10^7 Da).
253 These molecular masses are much larger than those measured in chloroform, indicating that ALDs form self-
254 aggregates in water, even at a low concentration of 0.5 g/L.
255

256 3.2. Enzymatic saccharification of unbleached softwood pulp in the presence of ALDs

257 Unbleached softwood pulp was enzymatically saccharified in the presence of EPEG-KL to determine its effect
258 on cellulase. Additionally, enzymatic saccharification was examined with PEG 4000, Tween 80 and the other ALDs,
259 the effects of which have already been investigated (Eriksson et al. 2002; Börjesson et al. 2007; Winarni et al. 2013,
260 2014). **Figure 4** shows the sugar yield and residual cellulase activity after enzymatic saccharification. All ALD
261 additives resulted in much higher sugar yields than the control (no additive), although PEG 4000 and Tween 80 gave
262 slightly higher yields than the ALDs. However, residual cellulase activities were maintained at much higher levels
263 by the ALD additives, especially in the case of EPEG-KL, compared to those of PEG 4000 and Tween 80 (**Figure**
264 **4-b**). In terms of ALD production, KL is easily isolated from black liquor in the kraft pulping process, which is the
265 most popular chemical pulping process worldwide. Therefore, EPEG-KL is easily prepared at a low production cost
266 compared to ALDs produced from SA and AL.

The order of residual enzyme activity depended on the PEG content in the ALDs; the lower the PEG content, the higher the residual cellulose activity. This study demonstrated that EPEG-KL is the most effective ALD for improving residual cellulase activity, and this effect can be attributed to the lignin moiety. The saccharification experiments were performed with ALD concentrations of 1.0 mg/mL, well above the concentration of 0.5 mg/mL at which the ALDs were found to self-aggregate in aqueous solution. These aggregates may also have contributed to the improved cellulase activity.

3.3. Estimation of interactions

3.3.1. Interaction of ALDs with hydrophilic functional group surfaces

Prior to the interaction of ALDs with cellulase components, the interactions between the ALDs and sensor chips adorned with hydrophilic functional groups were investigated to obtain fundamental information on the affinity of ALDs to electrostatic charges. **Figure 5** shows adsorption/desorption sensorgrams of the ALDs and PEG 4000 on hydroxy, carboxy and amino groups. In the sensorgrams, the analyte injection time was adjusted to 0, and 1 RU in the Y axis is equal to 1 pg/mm^2. Sensorgrams were classified into two types depending on the type of interaction and the strength of adsorption. The first type follows a trend wherein the adsorbed amount increases with an increase in the introduced amount and time of analyte, due to specific and/or strong interactions, similar to antigen-antibody or substrate-enzyme interactions. The second sensorgram type exhibits a rectangular shape, which is caused by a weak physical interaction (also called a bulk effect), such as a solvent-analyte interaction (Braslau et al. 1967). The sensorgram of EPEG-AL adsorbed on the amino group surface (**Figure 5A**) can be classified into the first type, which suggests a high affinity of the ALD for the cationic surface of the sensor chip. The other sensorgrams can be classified into the second type, indicating that the ALDs show a very weak affinity to anionic and non-ionic surfaces.

EPEG-SL and EPEG-KL (**Supplementary material**) showed tendencies similar to that of EPEG-AL. All sensorgrams of PEG 4000 showed a rectangular shape, indicating a weak interaction between PEG 4000 and the hydrophilic functional groups. The main structural difference between the ALDs and PEG 4000 stems from the lignin moiety. Therefore, the interaction between the ALDs and the amino groups can be attributed to an electrostatic interaction between the electronegative aromatic nuclei in lignin and the cationic amino groups (Ma and Dougherty 1997; Reddy and Sastry 2005; Mahadevi and Sastry 2013). In addition, isolated lignins generally have

295 carboxylate functionalities (Granata and Argyropoulos 1995), the content of which was not measured in this study.
296 The interaction between these carboxylic acids and the protonated amino groups may also influence the interaction
297 between the ALDs and the amino surface.
298

299 3.3.2. Interaction of ALDs with cellulase components CBH I, CBH II and EG I

300 In this study, three purified enzymes from a cellulase cocktail, CBH I, CBH II and EG I, were separately
301 immobilized on a Biacore sensor chip, using surface thiol coupling for CBH I and amino coupling for CBH II and
302 EG I. The amounts of immobilized enzymes were 2.35×10^4 RU (pg/mm^2) for CBH I, 1.35×10^4 RU for CBH II,
303 and 1.92×10^3 RU for EG I. Two other enzymatic components of cellulase, EG II and β-glucosidase, were not
304 investigated. Pure EG II could not be isolated due to a lack of abundance, and β-glucosidase is not a main
305 contributor to the cleavage of cellulose chains.

306 In the interaction analysis, the amount of adsorbed analyte on CBH I was calculated by subtracting the amount
307 of analyte adsorbed on the amino group surface from the apparent amount of analyte adsorbed on CBH I. Here, the
308 amino surface was used as a reference because it was generated by blocking excess thiol groups with PDEA (see the
309 conversion from compound 7 to 8 in Figure 2). Corrected sensorgrams for CBH I are shown in **Figure 6**. Similarly,
310 the amount of analyte adsorbed on CBH II and EG I was calculated by subtracting the amount adsorbed on the
311 hydroxy group surface from the apparent amount adsorbed on CBH II and EG I. Corrected sensorgrams for CBH II
312 and EG I are shown in **Figures 7** and **8**, respectively.

313 The amounts of all ALDs adsorbed on CBH I increased with prolonged exposure (**Figure 6a, b** and **c**),
314 suggesting a strong interaction, while the amount of adsorbed PEG 4000 did not show a time dependency (**Figure**
315 **6d**), indicating a weak interaction. This adsorption time dependency was also observed for EPEG-SL and -KL on
316 CBH II. The sensorgrams of EPEG-SL and -KL in **Figure 7** are similar to that of EPEG-AL, which was previously
317 reported (Winarni et al. 2013). These lignin derivatives showed a high affinity to CBH II. Again, PEG 4000
318 exhibited only a weak affinity to CBH II.

319 Interestingly, all sensorgrams of the ALDs as well as PEG 4000 for EG I binding (**Figure 8**) exhibited
320 rectangular shapes, although the adsorbed amounts were slightly increased at the end of the analyte introduction
321 period due to the instability of the analyzer used. PEG 4000 showed a much lower adsorption than the ALDs. Thus,
322 the ALDs and PEG all had very low affinities to EG I.

These studies on the interaction of ALDs and PEG 4000 with cellulase cocktail enzymes demonstrated the following results. The ALDs had strong affinities to CBH I and II, which are the dominant enzymes in cellulase secreted from *Trichoderma reesei* (Palonen et al. 2004), but the ALDs had a low affinity to EG I. The CBH group and EG I differ greatly in their substrate-binding domain. CBH I and CBH II have a tunnel structure that consists of four and two loops, respectively, which hold the substrate cellulose chain. EG I has no such tunnel (Börjesson et al. 2007a, b; Davies and Henrissat 1995), and cellulose chains are easily adsorbed and released by EG I. The ALD aggregates, which are formed in water by self-assembly at the 1 mg/mL concentration used for enzymatic saccharification, likely bind to the tunnel loop, loosening the tight binding to the cellulose chain. The binding between the ALD and enzyme is caused by hydrophobic and electrostatic interactions with enzyme surface amino groups, as mentioned above (Ma and Dougherty 1997; Reddy and Sastry 2005; Mahadevi and Sastry 2013). In addition, the ALD lignin moieties may inhibit the hydrophobic interaction between the CBH group and residual lignin in lignocellulosic substrates. This direct interaction of ALDs with the CBH group improved the sugar yield and residual cellulase activity in the enzymatic saccharification of unbleached pulp.

In contrast, PEG 4000 exhibited very weak affinities to all cellulase enzymes. This result suggests that PEG may associate with lignocellulosic substrates for improved saccharification and is consistent with previous reports on the interaction between PEG and lignocellulose (Börjesson et. al. 2007a, b). Because the residual post-saccharification cellulase activity was lower in the presence of PEG than in the presence of the ALDs, the direct interaction or association of additives with cellulase must be very important to the preservation of residual cellulase activity after enzymatic saccharification.

4. Conclusion

ALDs were prepared by the reaction of three types of isolated lignins with EPEG. Among the ALDs, EPEG-KL showed the highest residual cellulase activity. This result suggests that EPEG-KL is a promising cellulase-aid agent, especially in the process of ALD production, because KL is easily isolated from the black liquor of the kraft pulping process, which is currently the most popular chemical pulping process.

Our findings on the interactions of ALDs and PEG 4000 with cellulase cocktail enzymes demonstrate that the mechanism of improved enzymatic saccharification with ALD additives is quite different from that with PEG 4000.

The ALDs directly associated with CBH I (Cel7A) and II (Cel6A), but had a low affinity to EG I (Cel7B), while PEG 4000 did not show strong affinities to any cellulase enzyme. In addition, the ALDs exhibited significant interactions with amino groups. Consequently, the ALDs were strongly associated with CBH I and II due to their interactions with amino acids on the enzymes in addition to hydrophobic interactions.

Disclosures:

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Conflict of Interest: The authors declare that they have no conflict of interest.

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453

454 **Figure captions**

455 **Fig. 1** Chemical structure of 48,49-epoxy-3,7,10,13,16,19,22,25,28,31,34,37,40,43,46-
456 pentadecaoxanonatetracontane-5-ol (EPEG).

457 **Fig. 2** Sensor chip surface modification and immobilization of cellulase components. (a) Preparation of amino
458 groups (3) and hydroxy groups (4) on the surface of the sensor chip; (b) modification of CBH I for immobilization;
459 (c) immobilization of CBH I by surface thiol coupling; (d) immobilization of CBH II and EG I.

460 **Fig. 3** Size exclusion chromatograms of EPEG-SL in chloroform. A series of chromatograms was monitored on a
461 UV detector at 280 nm at concentrations of 0.5 mg/mL (a), 1.0 mg/mL (b), 2.0 mg/mL (c), and 3.0 mg/mL (d).
462 Chromatogram B was monitored on a MALS detector at a concentration of 3.0 mg/mL. In B, calibration curves of
463 absolute Mw and relative molecular mass were obtained by a direct measurement of the specimen using the MALS
464 detector and by using authentic polystyrene standards, respectively. The right Y-axis shows the molecular mass for
465 the calibration curves. (——SEC chromatogram, ……calibration for absolute Mw, ------calibration for relative
466 molecular mass)

467 **Fig. 4** Sugar yield (a) of the enzymatic saccharification of unbleached softwood kraft pulp and residual cellulase
468 activity (b) after saccharification in the presence of additives.
469 1, control (no additive); 2, PEG 4000; 3, Tween 80; 4, EPEG-AL; 5, EPEG-SL; 6, EPEG-KL.

470 **Fig. 5** Sensorgrams of EPEG-AL (A) and PEG 4000 (B) for various functional groups on sensor chips. (a) Hydroxy
471 group; (b), carboxy group; (c) amino group.

472 **Fig. 6** Sensorgrams of EPEG-AL (a), EPEG-SL (b), EPEG-KL (c) and PEG 4000 (d) for CBH I immobilized on the
473 sensor chip.

474 **Fig. 7** Sensorgrams of EPEG-SL (a), EPEG-KL (b) and PEG 4000 (c) for CBH II immobilized on the sensor chip.

475 **Fig. 8** Sensorgrams of EPEG-AL (a), EPEG-SL (b), EPEG-KL (c) and PEG 4000 (d) for EG I immobilized on the
476 sensor chip.

477

478

Table 1. ALDs characterizations.

ALDs	Elemental composition (%)				PEG content (%)	Mw[4] (g/mol)	M of Lignin[5] (g/mol)	N of PEG chains[6]
	C	H	N	S				
EPEG-AL	52.7	6.8	0.0	0.0	72.3[1]	1.61×10^4	4.47×10^3	15.6
EPEG-SL	59.0	7.3	0.0	0.0	63.5[2]	2.26×10^4	8.25×10^3	19.0
EPEG-KL	51.9	7.0	0.0	1.7	59.3[3]	2.32×10^3	9.45×10^2	1.8

[1]Winarni et al, 2013, [2]Cheng et al. 2014, [3]Aso et al. 2013, [4]Absolute Mw of ALDs estimated from MALS data, [5]Molecular mass of original lignin calculated from MALS data and PEG content, [6]the number of PEG chain per ALD molecule.

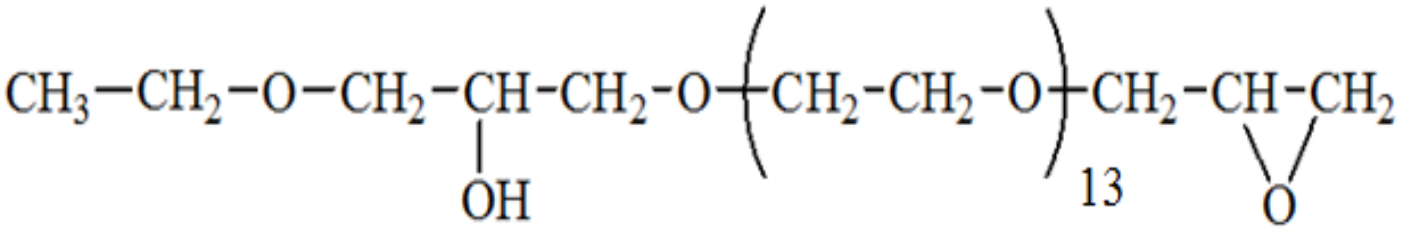


Fig. 1 Chemical structure of 48, 49-epoxy-3, 7, 10, 13, 16, 19, 22, 25, 28, 31, 34, 37, 40, 43, 46-pentadecaoxanonatetracontane-5-ol (EPEG)

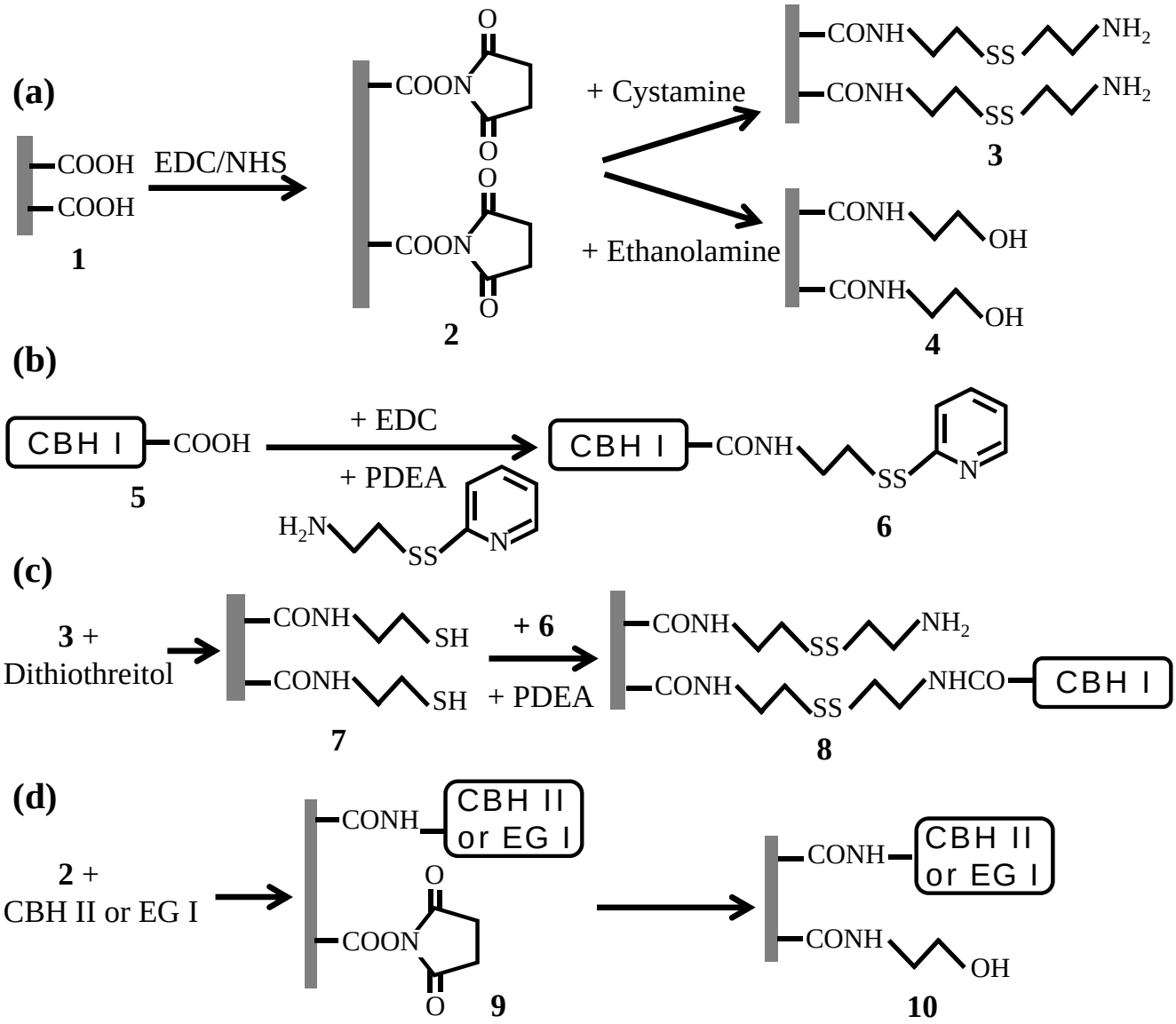
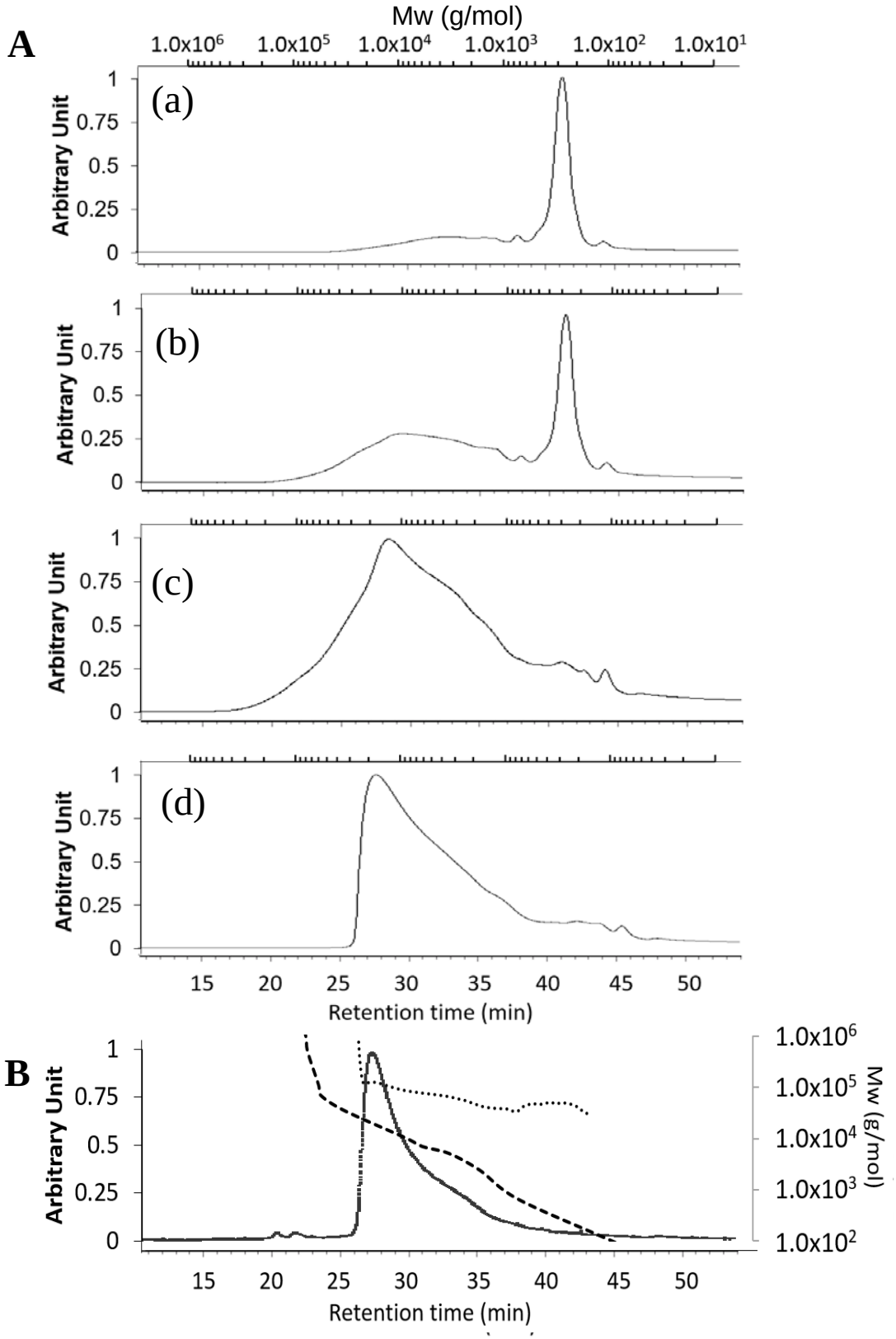


Fig. 2 Surface modification of sensor chip and immobilization of cellulase components on sensor chip. (a), preparation of amino group (3) and hydroxy group (4) on the surface of sensor chip; (b), modification of CBH I for immobolization; (c), immobilization of CBH I onto sensor by surface thiol coupling; (d), immobilization of CBH II and EG I on sensor chip



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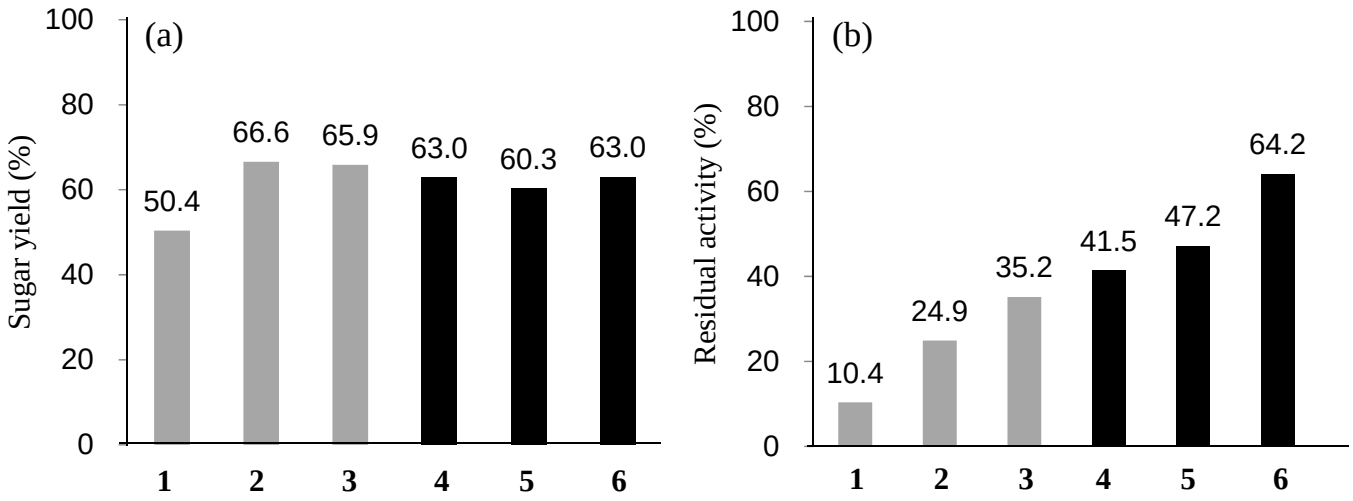
(——SEC chromatogram, ……calibration for absolute Mw, ------calibration for relative molecular mass)



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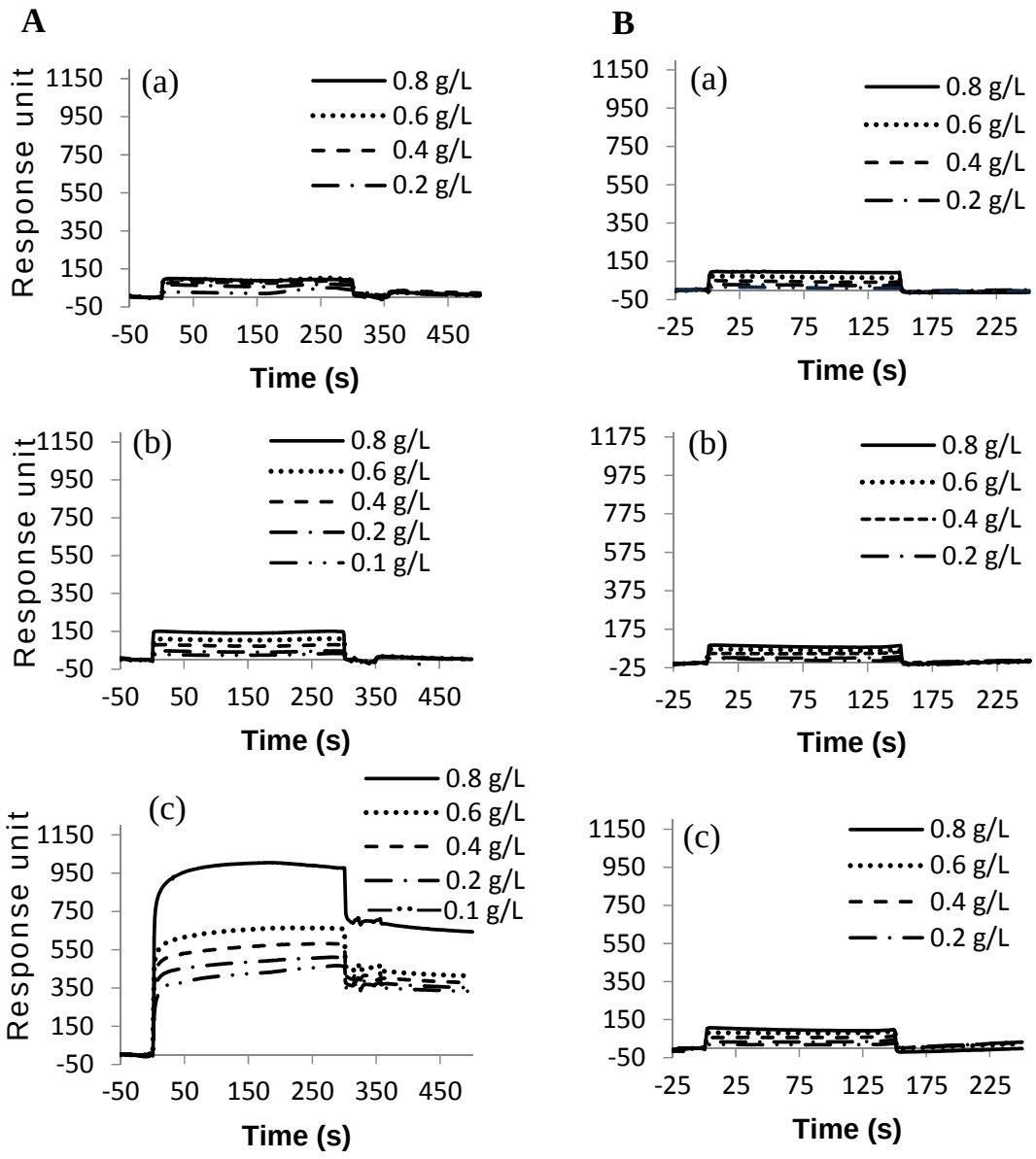

Fig. 5 Sensorgrams of EPEG-AL (A) and PEG 4000 (B) for various functional groups on sensor chips. (a), hydroxy group; (b), carboxy group; and (c), amino group

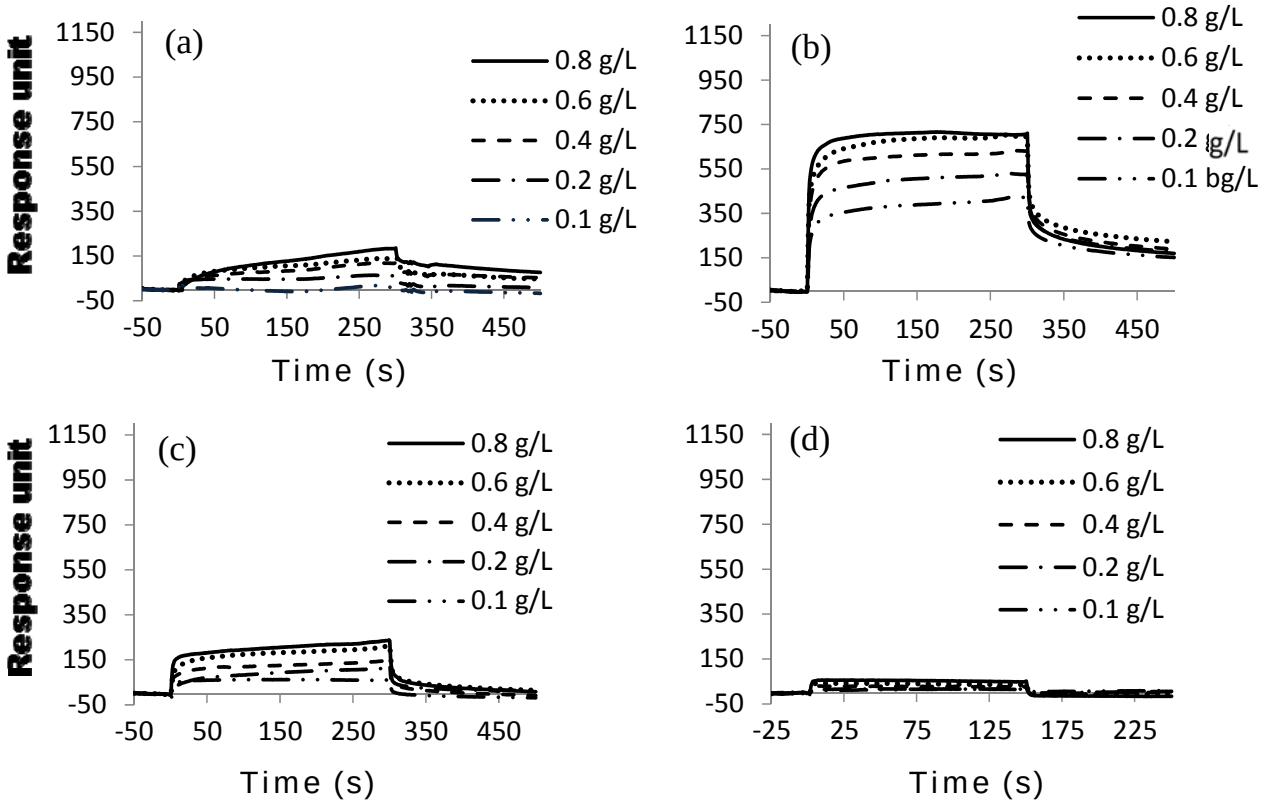

Fig. 6 Sensorgrams of EPEG-AL (a), EPEG-SL (b), EPEG-KL (c) and PEG 4000 (d) for CBH I immobilized on the sensor chip

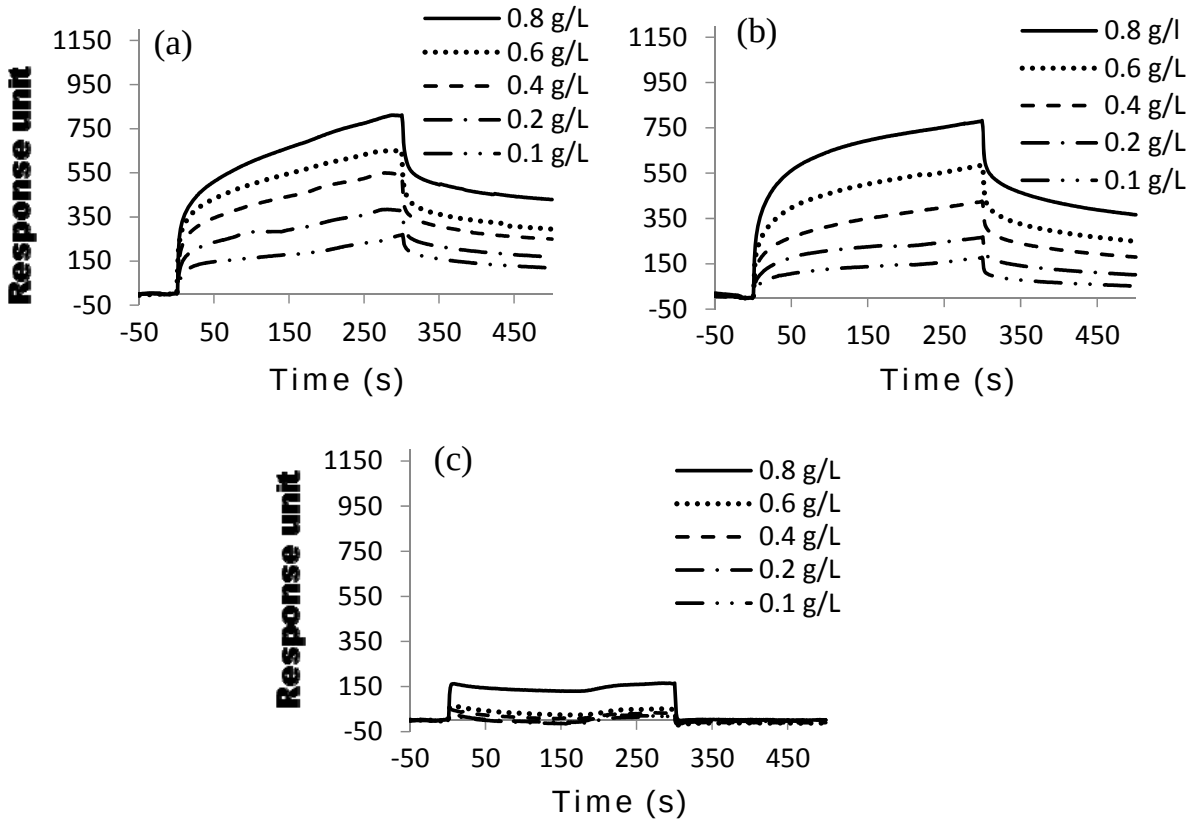

Fig. 7 Sensorgrams of EPEG-SL (a), EPEG-KL (b) and PEG 4000 (c) for CBH II immobilized on the sensor chip

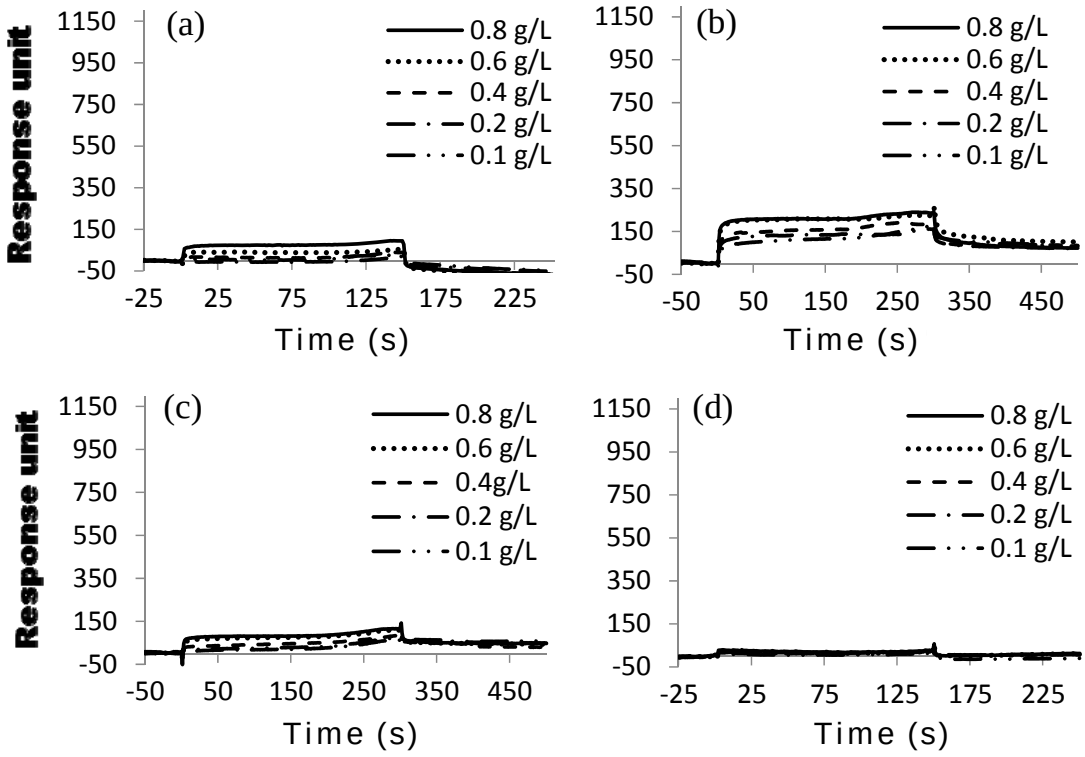

Fig. 8 Sensorgrams of EPEG-AL (a), EPEG-SL (b), EPEG-KL (c) and PEG 4000 (d) for EG I immobilized on the sensor chip