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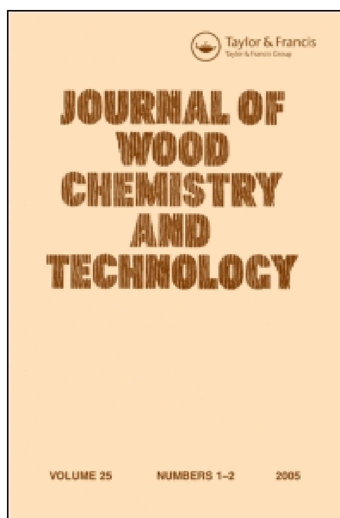

Journal of Wood Chemistry and Technology
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597282

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To cite this Article Uraki, Yasumitsu , Nakamura, Atsushi , Kishimoto, Takao and Ubukata, Makoto(2007) 'Interaction of Hemicelluloses with Monolignols', Journal of Wood Chemistry and Technology, 27: 1, 9 — 21
To link to this Article: DOI: 10.1080/02773810701282314
URL: http://dx.doi.org/10.1080/02773810701282314

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Journal of Wood Chemistry and Technology, 27: 9–21, 2007
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ISSN 0277-3813 print/1532-2319 online
DOI: 10.1080/02773810701282314

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Interaction of Hemicelluloses with Monolignols

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Abstract: Interactions of hemicelluloses, xylan and glucomannan, with monolignols, coniferyl alcohol and sinapyl alcohol, and their glucosides, coniferin and syringin, were investigated by surface plasmon resonance analysis. Hemicelluloses with different aldehyde contents, prepared by sodium periodate oxidation, were immobilized onto carboxymethyl–dextran–coated gold plates by reductive amination. The interaction of the hemicelluloses with monolignols was evaluated as weight gain of the hemicelluloses at neutral pH. Both hemicelluloses adsorbed the monolignols in much larger amounts than the corresponding glucosides. The adsorption tendency was independent of aldehyde content. These results suggest the hemicelluloses have higher affinities to the aglycons than the glucosides. Thus, the interactions of the hemicelluloses with monolignols are primarily the result of hydrophobic interactions. However, binding constants were not estimated because of the low solubility of the monolignols in the aqueous buffered solution.

Keywords: Hemicellulose, monolignol, monolignol glucoside, quantification of glucuronic acid, surface plasmon resonance (SPR)

Part of this work was financially supported by the Japan Society for the Promotion of Science (#15208016).

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INTRODUCTION

Elucidation of the lignification process is not only of increasing importance with respect to clarifying cell wall formation, but a necessity to enable improvements in chemical pulping. Within the last decade genetic engineering has dramatically enhanced our knowledge of monolingnol biosynthesis and its impact on pulping with regard to cost reduction and environmental load.[1–5] However, the lignification process, that is, post monolignol biosynthesis, is not yet clear.

The first stage of cell wall formation is cellulose deposition, followed by hemicellulose and lignin. Ligninification, or polymerization of lignin precursors, occurs throughout the hemicellulose phase, and results in the formation of lignin-carbohydrate complexes. Monolignols, coniferyl alcohol (CA) and sinapyl alcohol (SA), are generally accepted as the final precursors for lignification. However, how the precursors are transported from the cytosol through the plasma membrane to the hemicellulose matrix can be argued. Two hypotheses are proposed: One is that monolignol glucosides, coniferin (CR)[6–9] and syringin (SG),[10,11] are transported, and then are hydrolyzed by glucosidase to convert them to the corresponding aglycons. Finally, they are polymerized to lignin by enzymatic reactions. The other one is that monolignols are transported to the hemicellulose matrix, and are directly polymerized by enzymatic reactions. The basis of this hypothesis is due to an easy penetration of monolignols into plasma membrane.[12] If a better understanding of the interactions of monolignols or monolignol glucosides with hemicelluloses were ascertained, a better understanding of the lignification process may result.

Surface plasmon resonance (SPR) can be used to monitor the binding phenomena of monolignols and their glucosides to hemicelluloses. SPR is widely used to investigate interactions between two specific biomaterials, such as enzyme and substrate, antibody and antigen, and so on.[13–15] Recently, xylan adsorption onto multilayers of cellulose derivatives, which mimicked cell wall formation, was evaluated using SPR.[16] However, there is no report on the interaction between lignin precursors and hemicelluloses.

Using SPR, we can obtain physicochemical information about adsorption-desorption kinetics and binding constants using a small amount of sample and in a short period of time. In this analytical system the host molecule is termed ligand, and the guest molecule analyte. The ligand is immobilized on a solid support termed "sensor chip," and the analyte is pumped to the ligands in a buffer solution. The apparatus directly monitors the weight gain resulting from the association between the ligand and analyte. Similarly, dissociation between them was estimated as weight loss by washing with water or buffered solutions after the association. In our analytical system, hemicelluloses were used as ligands and were immobilized onto the sensor chip. Accordingly, the hemicelluloses in this system were suspension in a buffered solution or gel state. Monolignols (analyte) were introduced into the hemicellulose matrix. As a result the system closely resembles that of the lignification process, and mimics the encounter of monolignols with hemicelluloses in living tree.

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In this article, the interactions of lignin precursor candidates (monolignol and monolignol-glucoside) with hemicelluloses are reported. In addition, the immobilization process of hemicelluloses onto sensor chips is introduced. Furthermore, a modified method for the determination of uronic acids is also presented, as neutral monosaccharides were found to interfere with the determination of uronic acids when using the conventional protocol.[17]

EXPERIMENTAL

Materials

Hemicelluloses, birch xylan and Konjac glucomannan, and chemical reagents were purchased from Wako Pure Chemical (Osaka, Japan). Monolignols (CA and SA) and their glucosides (CR and SG) were synthesized according to previous reports.[18–20] These lignin precursor candidates, as shown in Figure 1, were used after purification by recrystalization. HBS-N buffer (10 mM HEPES, pH 7.4, containing 0.15 M NaCl) was purchased from Biacore (Tokyo, Japan). Milli-Q water was used in all measurements except for sample syntheses.

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Sugar Constituents in the Delignified Hemicelluloses

The xylan (20 g) with 0.6% Klason lignin was delignified with $NaClO_2$ (1.2 g) and acetic acid (1.2 ml) in 500 ml of deionized water for 1 h at 70°C. The glucomannan (15 g) with 1.0% Klason lignin was delignified with 2 g of $NaClO_2$ and 2 ml of acetic acid in 300 ml of deionized water. The delignified hemicelluloses were purified by dialysis against deionized water followed by lyophilization. Neutral sugar contents in the delignified hemicelluloses were determined by the alditol-acetate method[21] after hydrolysis with sulfuric acid.

Uronic acid content was determined by the method reported by Blumenkranz and Asboe-Hansen.[17] Briefly, $Na_2B_4O_7$ (1.2 ml, 12.5 mM) in

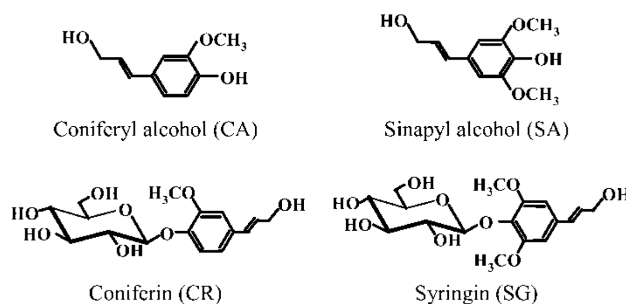

Figure 1. Chemical structure of monolignols and the corresponding glucoside analytes.

conc. H_2SO_4 was added to an aqueous hemicellulose solution (0.2 ml, 10 mg/ml) with gently stirring in ice bath. After cooling the reactants were thoroughly mixed with vigorous stirring, and then the hemicelluloses were hydrolyzed at 100°C for 5 min. The system was cooled in an ice bath and 20 μl of 0.15% *m*-hydroxydiphenyl in a 0.5% NaOH aqueous solution was added. The uronic acid content was calculated from the absorbance at 520 nm using a calibration curve that was made with authentic glucuronic acid, and corrected for neutral sugars; discussed in detail in the Results and Discussion section.

Immobilization of Hemicelluloses onto Sensor Chip

Carboxymethyl-dextran (CM-D) coated sensor chips, trade name CM-5, were obtained from Biocore (Tokyo, Japan), and used as the basic substrate. Immobilization of the ligands and SPR measurements were conducted on a Biocore™-X, Tokyo, Japan. The immobilization scheme is shown in Figure 2.

The delignified hemicelluloses were oxidized with 0.2–2.0 equivalents of $NaIO_4$ based on anhydropyranose unit. The resultant oxidized hemicelluloses were thoroughly dialyzed against deionized water, and then lyophilized to yield the modified hemicelluloses as specimens for immobilization. The extent of oxidation, aldehyde content, was determined according to a previously reported procedure.[22]

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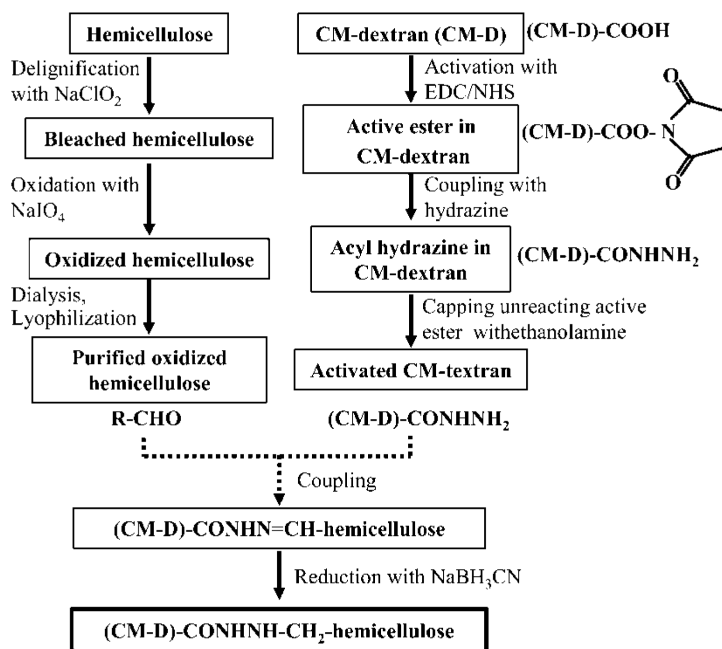

Figure 2. Preparation scheme for hemicellulose-immobolized sensor chip.

The carboxyl groups in CM-D were activated by reaction with a mixture (15 μl) of *N*-hydroxysuccinimide (NHS, 0.05 M) and *N*-ethyl-*N*-(3-dimethyl aminoisopropyl) carbodiimide (EDC, 0.2 M). The CM-D-NHS activated ester was then reacted with hydrazine (35 μl, 5 mM) in aqueous solution to give an acyl hydrazine. Coupling of the modified CM-D with the oxidized hemicelluloses was accomplished by repeated addition of an oxidized hemicellulose solution (35 μl), until the adsorption amount of the hemicelluloses onto CM-D was constant. The oxidized hemicellulose solutions were prepared by dissolving 5.0 mg of oxidized hemicellulose in 200 μl of water, followed by 40 μl of ethanol, 80 μl of dimethylsulfoxide and 320 μl of acetate buffer (pH4, 10 mM). Once saturation of the hemicellulose was obtained, the generated shiff-base was reduced with $NaBH_3CN$ (35 ml, 0.1 M) in an acetate buffer solution (pH4, 0.1 M). The resultant sensor chip was washed with 50 mM NaOH aqueous solution and HBS-N buffer until the SPR response was stable, to yield a hemicellulose-immobilized sensor chip in the measurement cell. The reference cell contained a CM-D-ethanol amine capped sensor chip. All agents in aqueous or buffered solutions were introduced to the sensor chip at a flow rate of 5 μl/min. The immobilization process was carried out at 25°C.

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Measurement of Adsorption Capacities of Monolignols and their Glucosides on Hemicelluloses

Monolignols and their glucosides (analytes) were prepared in HBS-N buffer in the concentration range of 0–25 mM. Adsorption of the analytes was performed by treating the sensor chip for 2 min at the flow rate of 20μl/min at 25°C. The analytes were then desorbed by washing with HBS-N buffer. As both cells (measurement and reference) were connected to the single flow line, the analyte solution and buffer were first introduced into the reference cell and then into the measurement cell. The amount of analyte adsorption–desorption was estimated by the difference in amounts measured between the measurement cell and reference cell using the Biocore™-X.

The hemicellulose-immobilized sensor chips were repeatedly used for the adsorption-desorption measurements after complete washing with 0.5 M NaOH and HBS-N buffer for 2 and 15 min, respectively.

RESULTS AND DISCUSSION

Sugar Constituents of Hemicelluloses

Commercially available birch xylan and Konjac glucomannan hemicelluloses were used in this study. It is reported that main chain structure of Konjac glucomannan is very similar to tree glucomannan.[23] The xylan and

glucomannan contained 0.6% and 1.0% lignin as Klason lignin, respectively. As an aim of this study is to elucidate the affinity between hemicellulose and lignin precursors, the hemicelluloses were delignified using sodium chlorite prior to SPR measurements. The resulting sugar constituents of the delignified hemicelluloses along with the neutral sugar contents are listed in Table 1.

In the process of uronic acid determination using the method reported by Blumenkranz and Asboe-Hansen,[17] it was found that neutral sugars also reacted with the reagent and slightly affected the optical measurement. To obtain a precise uronic acid content, we first made a linear calibration curve for each of the following sugars.

$$\text{For glucose (G)} : \text{G-Abs} = 1.71 \times 10^{-4}\text{C} + 0.056 - \text{B}$$

$$\text{For mannose (M)} : \text{M-Abs} = 2.58 \times 10^{-4}\text{C} + 0.053 - \text{B}$$

$$\text{For xylose (X)} : \text{X-Abs} = 8.11 \times 10^{-5}\text{C} + 0.059 - \text{B}$$

$$\text{For glucuronic acid (GU)} : \text{GU-Abs} = 5.34 \times 10^{-3}\text{C} + 0.055 - \text{B} \quad (1)$$

where Abs is absorbance at 520 nm, C is concentration of sugar (μg/ml), and B is the absorbance (0.062) for a blank experiment.

Based on the corresponding sugar contents (Table 1) that show only trace levels of others sugars, the absorbance for the two hemicelluloses are assumed as:

$$\text{Abs for xylan (AX)} = \text{X-Abs} + \text{GU-Abs} \quad (2)$$

$$\text{Abs for glucomannan (AG)} = \text{G-Abs} + \text{M-Abs} + \text{GU-Abs} \quad (3)$$

The absorbance for neutral sugars, such as G-Abs, M-Abs, and X-Abs, were calculated from their amounts determined by the alditol-acetate analysis. AX and AG are actual experimental values, and enable GU-Abs to be estimated from Eq. (2) and Eq. (3) Finally, the glucuronic acid content can be calculated in accordance with Eq. (1). Using these equations, the glucuronic acid content in xylan and glucomannan was determined to be 13.7% and 0%, respectively. In the absence of such correction, the uronic acid content of the glucomannan was determined to be several percent. These corrected values are in-line with reported values,[23,24] and are included in Table 1.

Table 1. Sugar constituents of birch xylan and conjac mannan

	Ara	Xyl	Man	Gal	Glc	Uronic acid
Xylan	1.8	84.1	0.2	0	0.2	13.7
Glucomannan	1.1	0	62.6	0.6	35.7	0

The values are percent.

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Preparation of Hemicellulose-Immobilized Sensor Chips

Although the esterification reaction between the hydroxyl groups of the sugars and the carboxyl groups of CM-D is likely to be an easy coupling method, it is not recommended because it is labile to alkaline conditions. Hence, two coupling protocols are proposed for immobilization of sugars onto CM-D. One is coupling the aldehyde group of the sugars with an acyl hydrazine of CM-D (Dextran-$CONHNH_2$) prepared by reductive amination. The other is to couple the carboxyl groups of CM-D with hydrazinized sugars (Sugar-CH_2NHNH_2), produced by reductive amination of sugar aldehyde groups with hydrazine. In both immobilization protocols, a key functional group is the aldehyde groups in the sugars. However, in hemicelluloses the only aldehyde group is at the reducing end of the rather large molecule.

We attempted to increase the aldehyde content of the hemicelluloses by oxidative cleavage of the C2-C3 bond in anhydromonosaccharide unit with sodium periodate. The results are shown in Table 2. Although a stoichiometric reaction, quantitative generation of aldehyde groups for both hemicelluloses as well as for starch was not obtained in our experiments. The oxidized hemicelluloses are denoted based on the hemicellulose and equivalents of sodium periodate used, for example xylan oxidized with 0.2 equivalent $NaIO_4$ is 0.2-XY, the corresponding glucomannan is 0.2-GM. The resultant hemicelluloses with two kinds of oxidation degrees were immobilized according to the first coupling protocol.

As shown in Figure 2, prior to immobilization, the carboxyl groups in CM-D were reacted with EDC and NHS. Then the activated NHS ester was reacted with hydrazine to give the resulting acyl hydrazine. Any unreacted activated NHS-ester was inactivated by reaction with excess ethanol amine.

Table 2. Di-aldehyde content in an anhydromonosaccharide unit generated by periodate oxidation

	Di-aldehyde content[b]		
$NaIO_4$ (eq.)[a]	Xylan	Glucomannan	Starch
0.2	0.08	0.20	0.08
0.5	0.35	0.33	0.23
1.0	0.60	0.48	0.52
2.0	0.70	0.69	0.56

[a]Equivalent of $NaIO_4$ charged to the number of anhydromonosaccharide unit.

[b]Di-aldehyde was generated at C-2 and C-3 positions in an anhydromonosaccharide unit. The aldehyde content in a unit is twice as much as the values in this table.

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The amine group of the acyl hydrazine was in turn coupled with an aldehyde group in the oxidized hemicellulose by reductive amination with $NaBH_3CN$. The immobilization amount of hemicelluloses was increased by increasing the introduction times of hemicellulose solutions (0.78 μg/100 μl/time) into CM-D on the sensor chips. After 3 times the amount of 0.2-XY and 0.2-GM were 3380 RU and 4060 RU, respectively (1 RU means one pg/mm^2 of sensor chip). For the 1.0-XY and 1.0-GM the amount of hemicellulose immobilized after 2 treatment times was 1860 RU and 1580 RU, respectively. No further immobilization of 1.0-XY and GM could be obtained with further additions. It is assumed that in the second addition of hemicellulose solutions all of the available amine groups in the acyl hydrazine reacted with the abundant aldehyde groups present in these highly oxidized hemicellulose samples.

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Adsorption of Monolignols and Their Glucosides onto Hemicelluloses Immobilized on CM-D

The adsorption behavior of the four analytes, CA, CR, SA, and SG were monitored by SPR over the concentration range of 0–25 mM (25 mM was the observed maximum concentration that all analytes completely dissolved in the HBS-N buffer). Figures 3 and 4 show sensorgrams for the adsorption-desorption process for each analyte. It is can be seen in all the sensorgrams that the introduction of analytes leads to a rapid increase in RU and formation of an equilibrium state. Likewise, desorption occurred rapidly. In fact quite often large vibrations occurred at the beginning of the desorption process as a reverse in the SPR signal occurred between the measurement cell and the reference cell [Figure 3(B), (D), and Figure 4 (D)]. The RU of the equilibrium state increased with increasing analyte concentration, suggesting that the monolignol analytes unambiguously adsorbed onto the hemicellulose ligand. However, despite the analyte adsorption-desorption being rapid, the trapezoid shape of the sensorgrams reveals the interactions of all of the analytes with the hemicelluloses are very weak.[25]

Figures 5 and 6 illustrate the adsorption capacity of the various ligands at various analyte concentrations. The adsorption capacity was calculated by dividing the adsorption amount (mmol), determined from the average of the plateau region in the sensorgrams, by the weight (g) of the immobilized hemicellulose.

It is apparent from Figures 5 and 6 that the CA and SA adsorption capacities are much larger than those of the corresponding glucosides, CR and SG, respectively, at all concentrations of analyte. The tendency of higher monolignol adsorption was independent of oxidized degree of hemicelluloses. These results suggested that both hemicelluloses had higher affinity toward monolignols than the corresponding glucosides.

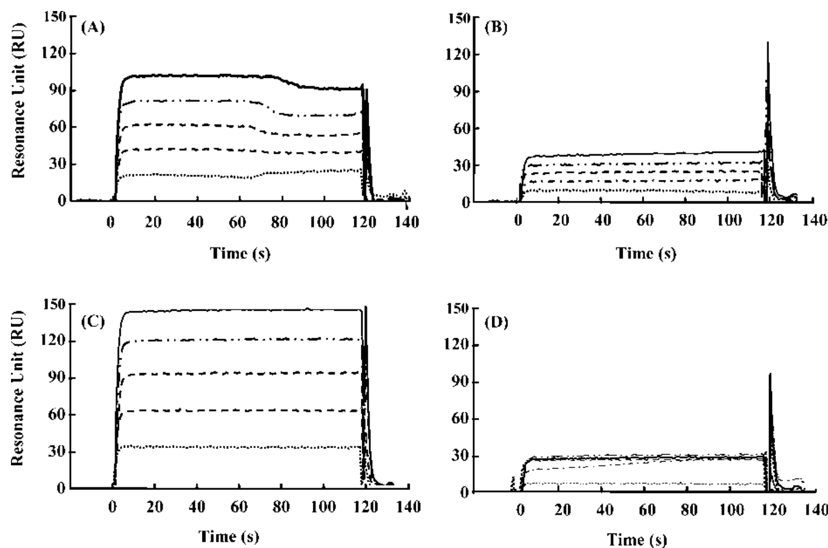

Figure 3. Sensorgrams of adsorption-desorption of CA (A), CR (B), SA (C), and SG (D) on 0.2-XY. Analyte concentration: 5 mM (......), 10 mM (— · —), 15 mM (– – – –), 20 mM (— · · —), and 25 mM (——).

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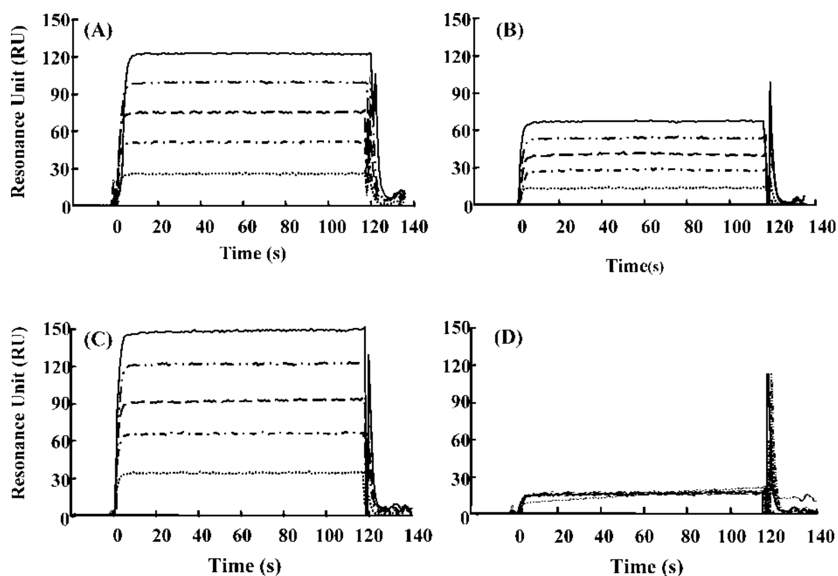

Figure 4. Sensorgrams of adsorptin-desorption of CA (A), CR (B), SA (C), and SG (D) on 0.2-GM. Analyte concentration: 5 mM (......), 10 mM (— · —), 15 mM (– – – –), 20 mM (— · · —), and 25 mM (———).

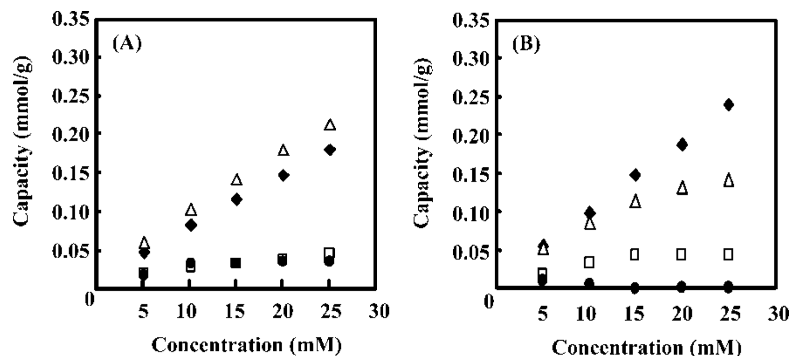

Figure 5. Adsorption capacity of CA (◆), CR (□), SA (△), and SG (●) on 0.2-XY (A) and 1.0-XY (B).

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Binding Constant

A binding constant or dissociation constant (*K*d) can readily be calculated from the result of SPR measurements. When an analyte strongly interact with a ligand, *K*d is calculated from the binding and dissociation rate constants, based on the time-dependency of adsorption and desorption amounts.[26] On the other hand, in the case of weak interactions the sensorgram shows a trapezoid shape, and *K*d can be calculated from the adsorption amount at different analyte concentrations and at the maximum adsorption using a Scatchard plot.[25] To estimate the maximum adsorption amount and *K*d it is necessary to have a saturated adsorption in the sensorgram.

For the monolignols (CA and SA) the adsorption capacities of both hemicelluloses, except SA on 1.0-XY (Figure 5(B)) increased proportionally with concentration. However, the adsorption did not reach saturated adsorption in

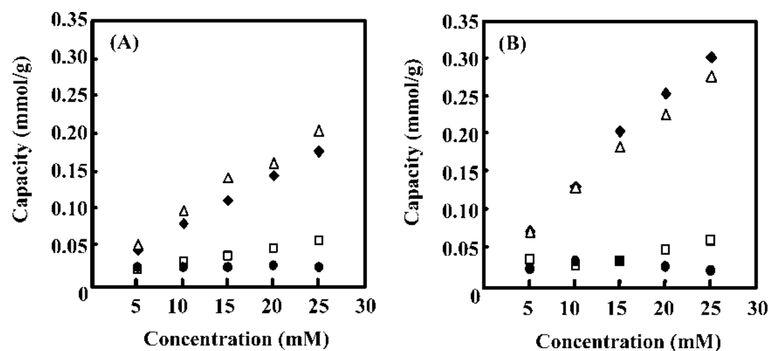

Figure 6. Adsorption capacity of CA (◆), CR (□), SA (△), and SG (●) on 0.2-GM (A) and 1.0-GM (B).

the range of measurement concentrations. Therefore, no binding (or dissociation) constant could be obtained. On the other hand, the adsorption capacity toward the glucosides (CR and SG) showed a saturation type profile [Figure 5(B)]. Although a calculation of the *K*d from such a profile using Scatchard plot is possible, we could not calculate reliable *K*d from the adsorption profile of the monolignol glucosides because of the small RU increment upon adsorption. For reliable calculation of *K*d, via a Scatchard plot, an RU variation of at least 20–30 is required. Nonetheless, we estimated the *K*d for the glucosides to be on the order of 10^{-3} M, which is consistent with weak binding. Furthermore, the *K*d values for the monolignols are predicted to be much smaller than the corresponding glucoside, assuming the maximum adsorption amounts are close to the values obtained at 25 mM analyte concentrations.

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CONCLUSIONS

Due to solubility issues, the *K*d for monolignols could not be obtained by SPR measurement. However, an obvious difference in the adsorption capacities between monolignols and their glucosides was confirmed; the adsorption capacities of monolignols were much larger than those of the corresponding glucosides. These results suggest that the hemicelluloses prefer hydrophobic compounds rather than hydrophilic ones; lignin precursor adsorption being mainly attributed to hydrophobic interactions.

On the basis of this conclusion, the following hypotheses are proposed. If monolignols are precursors they are readily transported from the cytosol to the site of lignification through the hemicellulose matrix by thermodynamically governed dispersion. This results, arising from the high affinity between the matrix and the monolignols as well as possible dispersion over cell membrane.[12] The dispersion of guest molecules into a polymer matrix is closely related to the affinity between the guest molecule and polymer.[27,28] A guest with high affinity easily penetrates and disperses into the matrix. Separation membranes are designed and prepared on the basis of such theory.[29,30] By contrast, if the precursors are monolignol glucosides, their transportations seems to require specific transporters such as proteins.

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