Effect of pH on the Formation of Benzyl Ester Bonds Between Glucuronic Acid and Dehydrogenation Polymer

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The effect of pH on the addition reaction of glucuronic acid to quinone methides generated in the synthesis of dehydrogenation polymer (DHP or artificial lignin) was investigated. The DHP-glucuronic acid complexes were formed during DHP polymerization catalyzed by a mixture of laccase, β-glucosidase, and O_2 within the pH range 7 to 4 in the presence of coniferin as a precursor. The structure of the product and the content of benzyl ester bonds were characterized by Fourier transform infrared spectroscopy, solid-state cross-polarization magic angle spinning carbon-13 nuclear magnetic resonance spectroscopy, ion chromatography, high performance liquid chromatography, and elemental analysis. The results showed that the pH of the reaction system had an important role in the formation of the benzyl ester bonds. Acidic conditions favored the reaction of quinone methide intermediates with carboxyl groups of glucuronic acid in the biosynthesis of DHP. However, weakly acidic conditions (pH 6) enhanced the reaction of quinone methide intermediates with glucose. In neutral conditions, the DHP-protein complex can be efficiently synthesized by the addition reaction of quinone methide intermediates with amino acids in protein.

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Keywords: Dehydrogenation polymer; Glucuronic acid; Quinone methides; Coniferin; Benzyl ester bond

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INTRODUCTION

Lignin is considered an amorphous and polyphenolic material arising from an enzyme-mediated dehydrogenative polymerization of three monolignol compounds, *i.e.*, coniferyl, sinapyl, and *p*-coumaryl alcohols. The polymerization of monolignols to form lignin comprises of three reaction steps: aroxyl radical formation *via* oxidation of monolignols; radical couplings leading to quinone methides that are an important intermediates for the biosynthesis of lignin; and nucleophilic addition reaction of quinone methides with nucleophiles during polymerization (Sarkanen and Ludwig 1971). Throughout lignification, a number of nucleophiles present in plants, such as water, alcohols, phenols, and carbohydrates, can react with quinone methides. For example, the addition of water leads to the formation of the β-O-4-structure, which is the most abundant structural unit in wood lignins (Adler 1977; Brunow *et al*. 1989). The addition of carbohydrates and lignin phenols can form lignin-carbohydrates and lignin-lignin complexes, respectively (Boerjan *et al*. 2003; Leary *et al*. 1978; Li *et al*. 2015; Ralph *et al*. 2001; Terashima *et al*. 1995; Xie *et al*. 2000). In addition, these nucleophiles will compete

for the addition reaction to quinone methides as well (Freudenberg and Neish 1968; Grabber *et al*. 2003). Therefore, the study of the reactions of quinone methides with these nucleophiles is important for fully understanding the various chemical bonds of lignin in plant.

Numerous studies with model compounds have demonstrated these competing reactions. Tanaka *et al* (1976, 1979) investigated the addition reactions of a quinone methide of dilignol with D-glucose and D-glucuronic acid in the presence or absence of water. They found that the presence of water markedly affects the reaction of D-glucuronic acid with quinone methides. The rate of the reaction between quinone methides and nucleophiles depended on the acidity and the steric factor of the nucleophiles (Tanaka *et al*. 1976, 1979). Sipilä and Brunow (1989, 1991a,b,c) investigated the addition reactions of a quinone methide of lignol with vanillyl alcohol, methyl-α-D-glucopyranoside, and D-glucuronic acid in water-dioxane solutions in various pH values. The results showed that the neutral conditions suppressed the addition reaction of water, while favoring the reaction of quinone methides with phenols from vanillyl alcohol. In contrast, the acidic conditions favored the reaction of quinone methides with water and uronic acids. In addition, methyl-α-D-glucopyranoside did not compete with phenols or water in reactions with the quinone methides either in neutral or in acidic conditions. These studies provide a lot of vital information about the addition reaction of quinone methides with nucleophiles. However, these addition reactions differed from the lignification of natural plant. To better understand the lignification process, many studies have focused on *in vitro* synthesis of artificial lignin, so-called dehydrogenation polymer (DHP), which was prepared by the polymerization of monolignols in the presence of hydrogen peroxide and peroxidase/ laccase, and oxygen (Freudenberg and Neish 1968; Terashima *et al*. 1995; Terashima and Atalla 1995; Cathala *et al*. 1998; Holmgren *et al*. 2009; Parijs *et al*. 2010; Hwang *et al*. 2015). It has been found that DHP is a good model compound for understanding the properties of native lignin and has been widely used to investigate the lignin structure and biosynthetic process.

The high mechanical strength and resistance to biodegradation of woody cell walls is thought to stem from the structure of lignin–carbohydrate complexes (LCCs) in wood (Toikka *et al*. 1998). LCCs from natural plant have important applications in materials and alternative medicine (Zhao *et al*. 2017a, 2017b; Sakagami *et al*. 2010). However, the isolation efficiency of LCCs from natural plant is not high, which limits its application. Artificial LCCs have the potential to greatly expand the application of LCCs. Lignin-carbohydrate benzyl ester is believed to be one of the main types of lignin-carbohydrate linkages in wood and has been suggested to be formed in reactions between acids groups of carbohydrates and quinone methides in the growing lignin macromolecule (Imamura *et al.* 1994). Coniferin, which occurs naturally in the cambial sap of gymnosperms and angiosperms, is considered to be an important precursor in lignin biosynthesis (Freudenberg and Harkin 1963; Tsuji *et al*. 2005). In the authors' previous study, lignin-pectin interactions were successfully modeled by performing *in vitro* oxidative polymerization of coniferin in the presence of pectin (Wang *et al*. 2013). The study focused on elucidating the impact of pH on the formation of the benzyl ester bonds during lignin biosynthesis, which provides theoretical guidance for preparation of artificial LCCs. The DHP was synthesized using coniferin as a precursor catalyzed by a mixture of laccase/β-glucosidase and O_2 in the presence of D-glucuronic acid in solution of various pH values. The contents of glucuronic acid, glucose, and CHON (carbon, hydrogen, oxygen, and nitrogen) -elements in the DHP was determined by ion chromatography (IC), high

performance liquid chromatography (HPLC), and elemental analysis. The structural characteristics of the products were investigated by Fourier transform infrared spectroscopy (FTIR) and solid-state cross-polarization magic angle spinning carbon-13 nuclear magnetic resonance (CP-MAS ^{13}C-NMR) spectroscopy.

EXPERIMENTAL

Materials

Coniferin was synthesized using a reported method of Xie and Terashima (1991). β-Glucosidase from almonds (specific activity: 6.3 units/mg), D-glucuronic acid, and 2,2’-azino-bis(3-ethylbenzthiazoline-6-sulfonate) (ABTS) were purchased from Sigma-Aldrich (Shanghai, China). Laccase (E.C.1.10.3.2) was donated by Novozymes A/S (Tianjin, China). The laccase used in this work was Novozym°51003 isolated from *Aspergillus oryzae*. All the chemicals used in this study were of analytical grade.

Methods

Enzyme assay

Laccase activity was determined *via* oxidation of ABTS. One activity unit (U) was defined as the amount of enzyme used to oxidize 1 μmol ABTS per minute in setting value buffer solution at room temperature (Aracri *et al*. 2011). To evaluate the effect of pH on laccase activity, the laccase activity was determined in buffer solution of sodium acetate/acetic acid (pH 4, 5, and 6) and in buffer solution of sodium phosphate (pH 7).

Preparation of DHP-glucuronic acid complexes (DHPGAC)

D-glucuronic acid (1.0 g) was dissolved in 50 mL buffer solutions of various pH values (0.2 M sodium acetate/acetic acid buffer solution for pH 4, 5, and 6; and 0.2 M sodium phosphate buffer for pH 7). Under sterile condition, β-glucosidase (100 mg) and laccase (715 U) were dissolved in sterilized water (3 mL) and were added to D-glucuronic acid solution. Coniferin (0.5 g) was dissolved in 50 mL of 0.2 M sodium acetate/acetic acid buffer solution (for pH 4, 5, and 6) and 50 mL of 0.2 M sodium phosphate buffer (for pH 7), respectively. Then, the coniferin solution was added dropwise to the mixture of D-glucuronic acid and enzymes within 24 h *via* a tube pump at 30 ℃ under the action of purified air bubbles. After 6 days, the crude product was obtained by centrifugation, followed by repeat-water wash and freeze drying.

Alkaline treatment of DHPGAC

Alkaline treatment of the prepared DHPGAC was performed following Xie *et al.* (2000). Approximately 100 mg of DHPGAC was dissolved in 1 N NaOH solution. The solution was stirred at 25 °C for 12 h under nitrogen atmosphere. After acidification with diluted HCl, the solution was made up to 50 mL with diluted H_2SO_4 and then centrifuged. The obtained precipitate was washed repeatedly with water and freeze-dried. The supernatant was evaluated to determine the content of D-glucuronic acid.

Determination of the content of D-glucuronic acid in DHPGAC

The supernatant obtained above was filtered through a 0.45-μm nylon filter and then analyzed by high-performance anion-exchange chromatography (HPAEC) (Dionex ICS-3000; (Dionex Corp., Sunnyvale, CA, USA) equipped with a CarboPacTM PA 20

column (3 × 150 mm^2), a CarboPacTM Guard column (3 × 30 mm^3), and a pulsed amperometric detection. The eluents used were pure water, 0.2 M NaOH, 0.5 M sodium acetate, and 0.02 M NaOH. The flow rate was 0.5 mL/min. The column temperature was 30 °C and the running time used was 30 min.

Characterization of the sample by infrared spectrometry

Infrared spectra of the samples were obtained using a Nicolet 380 FTIR spectrophotometer equipped with a DTGS detector (ThermoFisher Scientific Inc., Waltham, MA, USA). The FTIR analysis was performed on KBr pellets containing approximately 1.3 mg of sample for 180 mg of KBr. The number of scans used was 32, and the resolution was 4 cm^{-1}.

Structure characterization by CP/MAS ^{13}C-NMR spectra

The solid-state CP/MAS ^{13}C NMR spectra were recorded using an AV-III 400M spectrometer (Bruker Corp., Karlsruhe, Germany) at the frequency of 100.6 MHz with a 4 mm MAS-BBO probe. Acquisition time was 0.02 s. The delay time was 3 s and the proton 90° pulse time was 3 s. Each spectrum was obtained with an accumulation of 3600 scans. For purposes of comparison, the spectra were normalized on the basis of the resonance at 112 ppm, which is assigned to C-2 of the guaiacyl units (Terashima *et al*. 1995).

Determination of CHON-elemental contents

The CHON-elemental content analysis in DHPGAC was performed with a Vario EL cube elemental analyzer (Elementar Analysensysteme GmbH, Hanau, Germany).

RESULTS AND DISCUSSION

Content of Benzyl Ester Bonds in DHPGAC

Benzyl ester bonds in DHPGAC complex can be completely broken after sufficient alkali treatment, releasing glucuronic acid to the solution. Free glucuronic acid present in the complex had been removed before alkali treatment. Therefore, the content of glucuronic acid in the alkali-treated solution was only from the breakage of benzyl ester bonds of DHPGAC. The content of glucuronic acid was related to the amount of the benzyl ester bonds in DHPGAC and was obtained by IC analysis, as shown in Fig. 1.

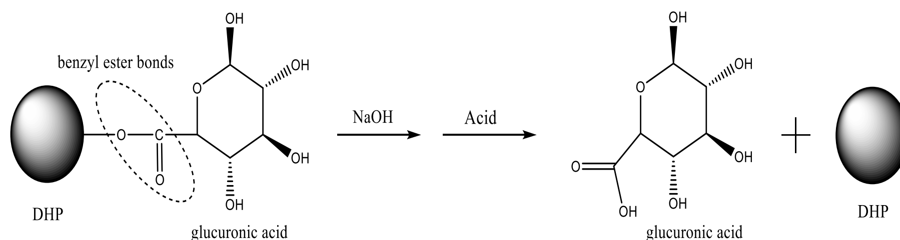

Fig. 1. Cleavage of benzyl ester bonds from DHPGAC through alkali treatment

The molar content of benzyl ester bond was expressed by the molar content of glucuronic acid. It can be seen from Table 1 that the pH of the reaction medium had an important role in the formation of benzyl ester bonds between glucuronic acid and DHP. The content of benzyl ester bonds decreased noticeably with increasing pH from acidic to

neutral. The result is in good agreement with the reports stated by Sipilä and Brunow (1991c). The results also revealed that the content of benzyl ester bonds formed between glucuronic acid and DHP under acidic or neutral conditions was low, which may be related to the addition reaction of water with quinone methide intermediate under acidic conditions (Sipilä and Brunow 1991a).

Table 1. Content of Benzyl Ester Bonds in DHPGAC at Various pH Values

pH Values	4	5	6	7
Benzyl Ester Bonds (μmol/g)	8.162	3.516	3.697	2.339

Content of CHON Elements in DHPGAC

There is evidence that lignin developing in cell walls enters into covalent bonds with cell-wall protein (Whitmore 1982). However, direct evidence of the covalent bonds is not available. Diehl and Brown (2014) investigated the cross-linking of various nucleophilic amino acids with DHP under aqueous conditions. They found that DHP-protein complexes were formed through the reaction of acid amino with quinone methides. The results of these studies may provide direct evidence of covalent bonds between phenolic structures and protein. The elemental contents in DHPGAC are shown in Table 2. It can be seen that there was a little change in the total elemental content in DHPGAC at various pH values. However, the N element content increased with increasing pH from acid to neutral, reaching the highest level under neutral condition. Because of the participation of in the synthesis of DHPGAC, the enzymes could be incorporated into DHPGAC through the addition reaction of proteins from the enzymes with DHP. The proteins in the enzymes may be the source of the nitrogen. The data in Table 2 also showed that the addition reaction of proteins with DHP was greatly influenced by the pH value of the reaction medium. The addition reaction of proteins with DHP was more favorable at higher pH value. The neutral condition can favor the reaction of DHP with various proteins from enzymes, leading to a negative impact on the synthesis of benzyl ester bonds. This is mainly due to the reaction between the amino acids quinone methides with varying efficiencies (in general, amine>thiol>acid>hydroxyl) under neutral conditions (Diehl *et al.* 2014). Therefore, amino groups in proteins are more likely to react with quinone methides during the formation of DHP under neutral conditions.

Table 2. CHON Elemental Contents in DHPGAC

pH Value	N (%)	C (%)	H (%)	S (%)	O (%)
4	1.98	59.00	6.19	0.00	32.89
5	2.27	58.75	6.17	0.00	32.56
6	3.36	54.36	6.62	0.00	34.06
7	4.36	53.17	6.37	0.11	32.81

Content of Glucose in DHPGAC

The glucose content present in the molecular structure of coniferin is released by the action of β-glucosidase, as shown in Fig. 2. To investigate the reaction of glucose with DHP, the glucose content in DHPGAC was determined. It can be seen from Table 3 that the pH of the reaction medium had a great impact on the glucose content in DHPGAC. Under the condition of pH 6.0, the glucose content in DHPGAC was the highest. The

results showed that glucose was easily added to the quinone methides during the formation of DHP at pH 6.0.

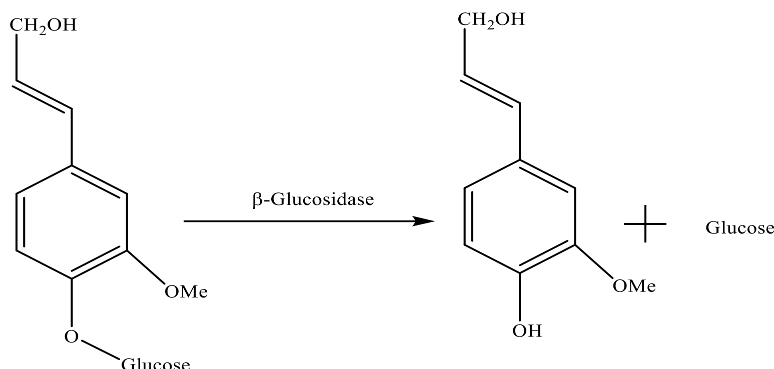

Fig. 2. Enzymolysis of coniferin

Table 3. Content of Glucose in DHPGAC at Various pH Values

pH Values	4.0	5.0	6.0	7.0
Glucose contents in DHPGAC (%)	0.319	0.087	4.626	1.102

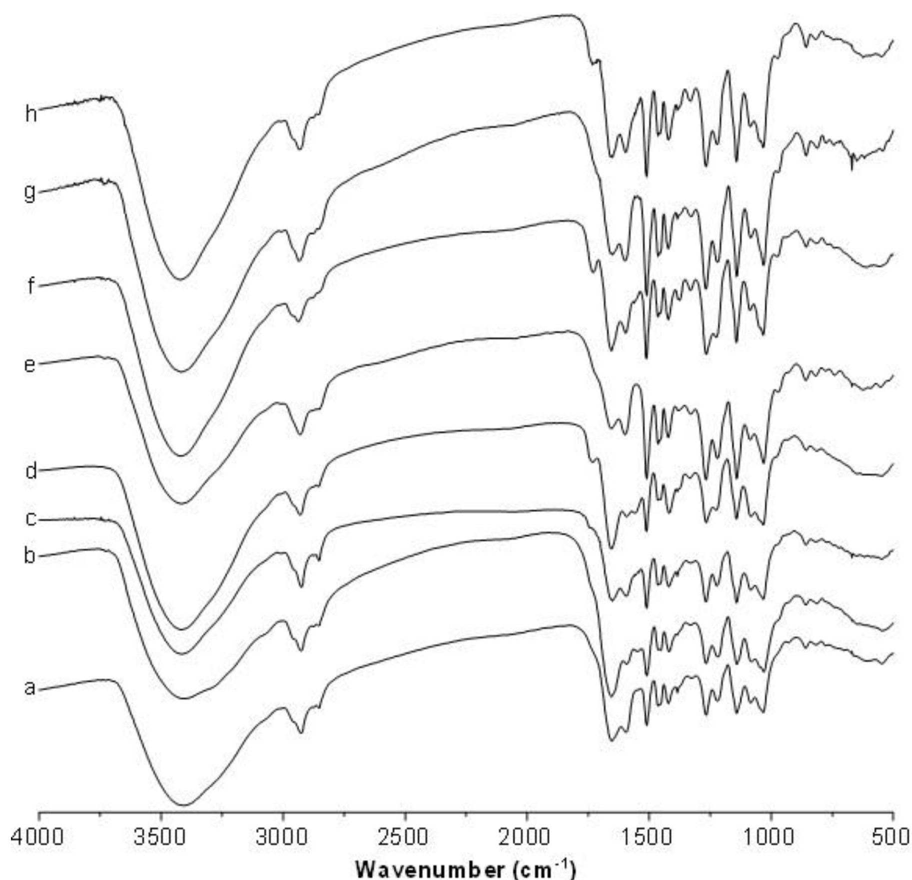

Fig. 3. FTIR spectra of DHPGAC: (a) pH 7 (treated with alkali); (b) pH 7; (c) pH 6 (treated with alkali); (d) pH 6; (e) pH 5 (treated with alkali); (f) pH 5; (g) pH 4 (treated with alkali); (h) pH 4

FTIR Spectra of DHPGAC

Figure 3 shows the IR spectra of DHPGAC before and after alkali treatment at various pH values. It can be seen that the DHPGAC prepared under acidic (pH 4, 5, and 6) and neutral (pH 7) conditions exhibited characteristic absorption peaks at 1592, 1511, and 1421 cm^{-1} of the aromatic skeletal vibration, and the intensity of absorption peaks was the same. It can also be seen that DHPGAC prepared under acidic conditions had an obvious absorption peak at 1731 cm^{-1}, which might be assigned to the absorption of carboxyl groups and ester bonds. However, the absorption peaks at 1731 cm^{-1} disappeared after alkali treatment. These results revealed that the absorption at 1731 cm^{-1} was mainly associated with ester bonds, indicating that benzyl ester bonds were formed between glucuronic acid and DHP under acidic conditions. There was no absorption peak at 1731 cm^{-1} for DHPGAC prepared under neutral (pH 7) conditions either before or after alkali treatments, indicating that there were no or minimal ester bonds present in DHPGAC. This agrees with the conclusion about the content of benzyl ester bonds mentioned above. Figure 3 also shows that the absorption peak of DHPGAC at 1654 cm^{-1} was gradually enhanced with the pH increase from acidic to neutral condition. The DHPGAC prepared at pH 7 also showed an absorption peak at 1546 cm^{-1}. These can be assigned to the amide bands (McDougall *et al*. 1996). These absorbances were due to the incorporation of proteins in enzymes into DHPGAC. This finding is consistent with the results of elemental analysis.

Table 4. Assignment of FTIR Spectra of DHPGAC

Wavenumber (cm^{-1})				Assignment
pH 4	pH 5	pH 6	pH 7	
3420	3416	3417	3405	O–H Stretch (H-bonded)
2932	2937	2930	2926	C–H Stretch in methyl and methylene groups
1731	1727	1731	—	C=O Stretch of unconjugated carbonyls
1650	1654	1654	1654	N-H Stretch in amide C=O Stretch of conjugated carbonyls; and C=C Stretching of coniferyl alcohol
1595	1596	1592	1592	C=C Aromatic skeletal vibration
1510	1511	1511	1508	C=C Aromatic skeletal vibration
1463	1463	1463	1453	C–H Bendings of $-OCH_3$ and $-CH_2$ groups
1421	1421	1418	1419	Aromatic skeletal vibrations combined with C–H in-plane deformation
1384	1375	1384	1384	C–H Stretch in $-CH_3$; O–H Stretch in aromatic skeleton
1331	1330	1332	1330	S-ring plus G-ring condensed
1267	1265	1266	1266	G-ring, and -C=O stretchings
1221	1225	1225	1218	-C–C, -C–O, and -C=O stretchings
1142	1142	1142	1140	Aromatic -C–H in-plane deformation
1085	1086	1082	1080	-C–O Deformation in secondary alcohols and aliphatic ethers
1032	1033	1032	1030	-C–O Stretch of methoxy groups of G-rings
856	856	856	857	-C–H Out-of-plane deformation (one H, aromatic ring)

CP/MAS ^{13}C-NMR of DHPGAC

The CP/MAS ^{13}C-NMR analysis was performed to characterize the chemical structure of DHPGAC and is shown in Fig. 4.

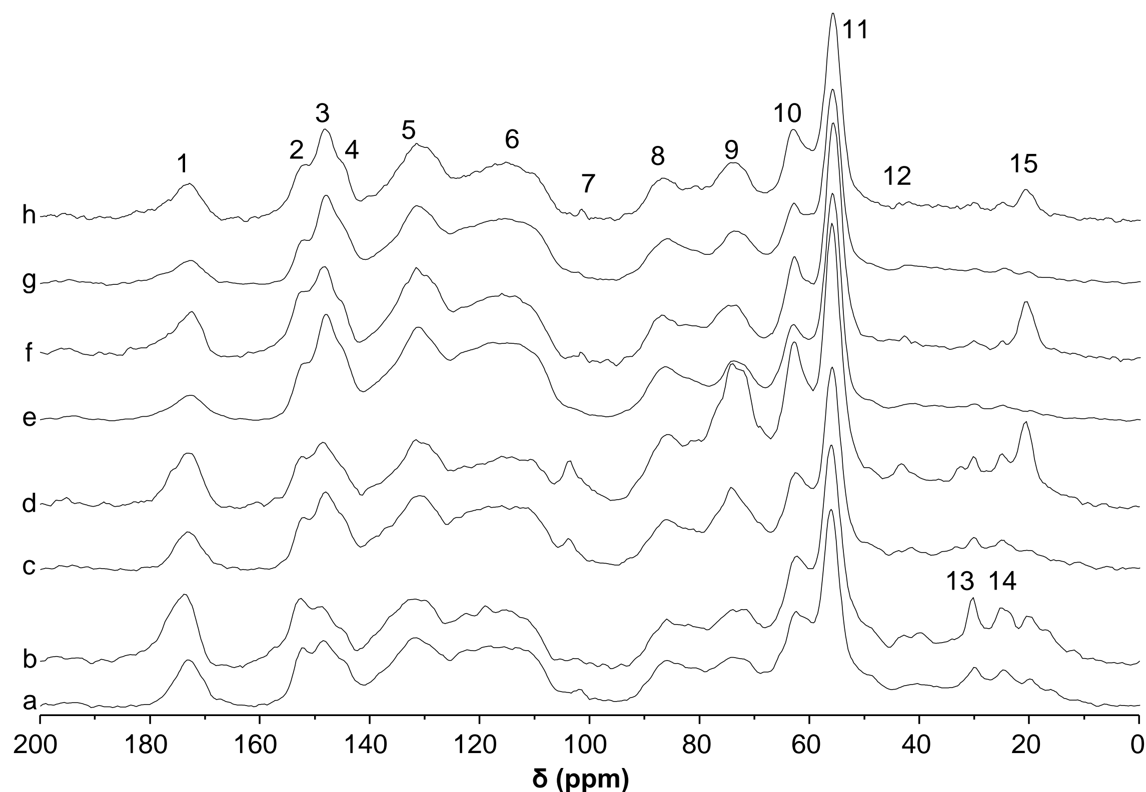

Fig. 4. CP/MAS ^{13}C-NMR spectra of DHPGAC: (a) pH 7 (treated with alkali); (b) pH 7; (c) pH 6 (treated with alkali); (d) pH 6; (e) pH 5 (treated with alkali); (f) pH 5; (g) pH 4 (treated with alkali); (h) pH 4

Table 5. Chemical Shifts and Assignments of Major Peaks in the CP/MAS ^{13}C NMR Spectra of DHPGAC

Peak No.	Chemical Shifts (*δ*, ppm)				Assignments
	pH 4	pH 5	pH 6	pH 7	
1	172.7	172.7	172.8	173.8	-COO- in aliphatic esters and acetyl group
2	152.1	151.9	152.7	152.7	C3/C4 in G-ring
3	148.3	148.5	148.9	149	C3/C5 in G-ring
4	147.1	147.4	147.4	147.3	C4 in G-ring
5	131.2	131.5	131.1	131.6	C1 in G-ring
6	119.1	119	118.9	118.7	C6 in G-ring
7	88.3	87.9	87.7	88.6	Cα(β-5, β-β)
8	80.5	81.1	80.8	81.2	Cβ in β-O-4
9	74	74.3	74.3	74.3	Cα in β-O-4, Cα of ester
10	62.9	62.6	62.8	62.8	Cα/Cβ in β-1, Cγ in β-5, and β-O-4
11	54.7	54.7	54.8	56.9	$-OCH_3$
12	42.1	42.6	43.2	42.9	α-CH_2- Side chain of phenylpropane units
13	30.9	29.9	30.9	30.4	β-CH_2- Side chain of phenylpropane units
14	24.1	24.5	24.8	24.1	$-CH_2$ in Saturated alkyl
15	20.7	20.6	20.4	20.1	$-CH_3$ of acetyl

It can be seen from Fig. 4 that the intensity of the peak at 172 ppm assigned to carbonyl groups of acetoxy and ester groups in the spectra of DHPGAC decreased after alkali treatment, which may be due to the breakage of benzyl ester bonds between

glucuronic acid and DHP. This further confirmed that benzyl ester bonds were formed between DHP and glucuronic acid. Strong peaks at 103 ppm and 74 ppm were also observed in the spectrum of DHPGAC prepared under pH 6 condition. These signals were assigned to the various carbons of glucose, namely C-1 (103 ppm) and C-2/C-3/C-5 (74 ppm). This indicated that the DHPGAC prepared under pH 6 condition contained a large amount of glucose.

CONCLUSIONS

1. During the synthesis of dehydrogenation polymer (DHP), which has been called artificial lignin, the intermediate quinone methides formed easily react with glucuronic acid to form benzyl ester bonds under acidic condition. The most favorable condition was at pH 4.
2. Total elemental analysis showed that DHP-glucuronic acid complexes (DHPGAC) contained N element. The content of N element increased with the increase of pH from acidic to neutral, which indicated that amino groups in proteins were more likely to react with quinone methides during the formation of DHP under neutral conditions.
3. The DHPGAC prepared under pH 6 condition contained more glucose, which indicates that glucose can be efficiently added to the quinone methide intermediate produced during the formation of DHP at pH 6.

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