SYNTHESIS AND CHARACTERIZATION OF PECTIN-DEHYDROGENATION POLYMER COMPLEX BY ISOTOPIC LABELING METHOD

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Coniferin is closer to natural lignin precursors than coniferyl alcohol. Both conventional and α-^{13}C labeled coniferin were polymerized in the presence of pectin to obtain a pectin-dehydrogenation polymer (pectin-DHP) complex under a closer approximation condition of the natural process for the formation of macromolecular lignin in the cell wall. A pectin-DHP complex was isolated and purified by extraction, hydrolysis, centrifugation and gel-adsorption method. The structure of the complex was analyzed by ^{13}carbon nuclear magnetic resonance (^{13}C-NMR) spectroscopy. The results of ^{13}C-NMR spectroscopy indicate that DHP was covalently linked to the pectin by benzyl ether bonds, ester bonds and acetal bonds. It was also found that the structure of the pectin-DHP complex approximated that of native lignin more closely than did the structure of the DHPs prepared from coniferyl alcohol. By these results, it was demonstrated that the lignin-pectin complex occurs in plant cell during lignin formation.

Keywords: pectin, coniferin, isotopic labeling method, ^{13}C-NMR

INTRODUCTION

Pectin, located in the primary wall of the plant cell, is a kind of polysaccharide.[1] It is commonly assumed that lignin polymer is bound covalently to polysaccharides to form lignin-carbohydrate complexes (LCCs).[2-5] Consequently, the structure of LCC from lignin and pectin may be formed during lignin biosynthesis. It is well known that lignin and LCC are macromolecular structures and are difficult to separate completely from the cell wall in plants. Therefore, little direct evidence of the linkage between lignin and pectin has been obtained so far. At present, Dehydrogenation Polymers (DHPs) of monolignols are usually used as lignin model compound to understand the structure of native lignin.[6] Therefore, artificial LCC, i.e., pectin-dehydrogenation polymer (pectin-DHP) complex may be synthesized and resembles the LCC model compound. Higuchi *et al*.,[7] Terashima *et al*.,[8] and Cathala *et al.*[9,10] synthesized the pectin-DHP complex with coniferyl alcohol and pectin by the action of peroxidase in the presence of H_2O_2. Higuchi and Terashima studied the molecular weight and structure of pectin-DHP complex and indicated that DHP was connected with pectin. Cathala had confirmed the occurrence of ester bonds between pectin and DHP by alkaline and acidic degradation. In the studies mentioned above, coniferyl alcohol was used as lignin precursor. The structure of DHP from the pectin-DHP complex may be quite different from the structures of native lignin, because lignin precursors should be in the form of glycosides in plants.[8]

Coniferin is a kind of monolignol glucoside and occurs naturally in the cambial sap of gymnosperms and angiosperms. It is considered to be a very important precursor in lignin biosynthesis.[11,12] Freudenberg anticipated that enzymatic hydrolysis of monolignol glucosides, such as coniferin, liberate the monolignols, which subsequently undergo dehydrogenative polymerization to form protolignin macromolecules in the cell walls.[13] In our previous work, guaiacyl type DHP-holocellulose complex was prepared from holocellulose and coniferin by horseradish peroxidase, β-glucosidase and glucose oxidase and it was found that the structure of the

DHP-holocellulose complex was very similar to gymnosperm (ginkgo woods) xylem tissue.[14]

^{13}Carbon-NMR spectroscopy has been shown to be a powerful non-destructive method for the structural analysis of lignin, but it faces serious problems in the assignments of signals of lignin carbons because of the low natural abundance of the ^{13}C-isotope and the overlaps of carbohydrate and lignin signals in NMR.[15] Therefore, an isotopic labeling method was used to determine the structure of lignin by enhancing the signal of a specific carbon in lignin or DHP-holocellulose complex with ^{13}carbon.[14-21] In the present work, coniferin and coniferin-[side chain-α-^{13}C] were synthesized and pectin-DHP complexes were prepared from coniferin and coniferin-[side chain-α-^{13}C] in the presence of pectin, the reaction being catalyzed by horseradish peroxidase, β-glucosidase and glucose oxidase. Then, a ^{13}C isotopic labelling method in combination with NMR for the study of pectin-DHP complex structures and reactions was applied. Structural information on pectin-DHP complexes will be beneficial for understanding the connection of lignin with pectin in plant cell wall.

EXPERIMENTAL

Materials

β-Glucosidase from almonds (specific activity: 6.3 units/mg), glucose oxidase (Type II: from *Aspergilus niger*, specific activity: 15,500 units/g) and peroxidase (Type II: from horseradish, specific activity: 158 purpurogallin units/mg) were purchased from Sigma-Aldrich. Pectinase (Pectinex BE XXL, specific activity: 16,000 units/mL) was purchased from Novozyme. Pectin from citrus peel (galacturonic acid, 74%) was also purchased from Sigma-Aldrich.

Preparation of coniferin

Both coniferin-[side chain-α-^{13}C] and unlabelled coniferin were prepared following the procedure developed by Terashima,[22] as shown in Fig. 1 The start material was $CH_3{}^{13}COONa$ with 99% of ^{13}C isotope abundance. The melting point of the obtained coniferin-[side chain-α-^{13}C] was 184-185 ºC.

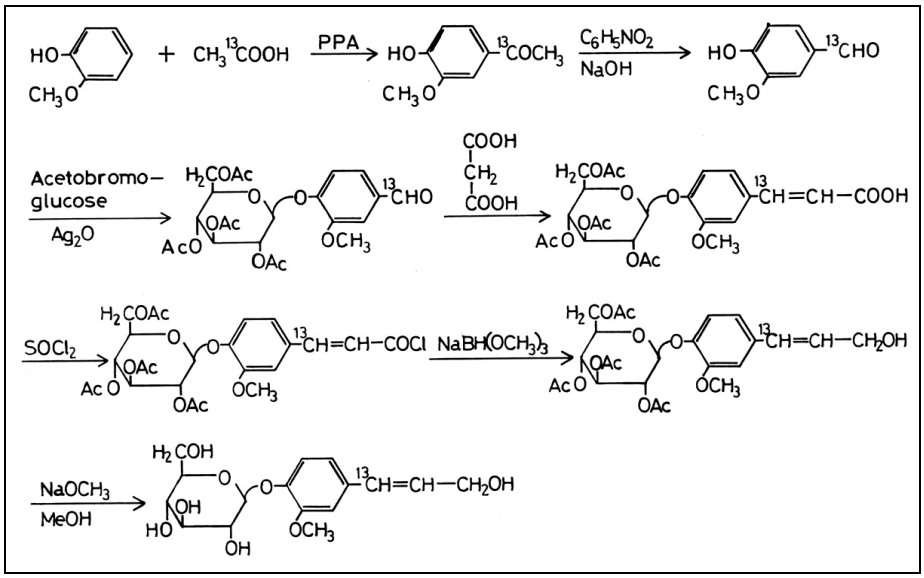

Figure 1: Synthesis of coniferin-[side chain-α-^{13}C]

Preparation of pectin-DHP complex

Pectin (1500 mg) was gelatinized by stirring in hot distilled water (100 mL). When the solution was cooled to ambient temperature, a pectin gel was obtained. β-Glucosidase (42.6 mg), glucose oxidase (72.6 mg) and peroxidase (1 mg) were dissolved in 3 mL of sterile water and then the solution was added to the pectin gel under sterile conditions. A mixture of coniferin-[side chain-α-^{13}C] (300 mg) and unlabelled coniferin (1200 mg) was dissolved in sodium acetate/acetic acid buffer solution (0.2M solution, pH 5.0, 100 mL). Afterwards, the coniferin solution was dropped to the mixture of pectin and enzymes using a tube pump at 26 -30 °C within 24 h under the action of purified air bubbles. After the addition of coniferin, the reaction continued to progress for 10 days at 26-30 °C with air bubbling. The crude product was obtained by vacuum evaporation and freeze-drying. After that, the crude product was extracted by dioxane/water (96/4, v/v) to remove free DHPs without linkages to pectin and the ^{13}C-labelled pectin-DHP complex was obtained (coded PDHPC-[α-^{13}C]).

A procedure for preparation of unlabelled pectin-DHP complex (coded PDHPC-cont.) Except for the use of unlabelled conifein (1500 mg), almost identical method above was used.

Enzymatic degradation of PDHPC and isolation of water-insoluble and water-soluble PDHPC

In order to remove the pectin without linkages to DHP, the pectin-DHP complex was hydrolyzed by pectinase. The PDHPC (3000 mg) was suspended in a filtered pectinase solution in sodium acetate/acetic acid buffer solution (0.2M, pH 4.0, 30 mL pectinase solution in 100 mL buffer solutin). The enzymolysis of PDHPC suspension was carried out on a shaker at 50 °C for 24 h. The enzymatic degradation products of PDHPC were separated into water-insoluble fractions and water-soluble fractions by centrifugation. Water-insoluble fractions were washed with distilled water to remove degraded glucose and then freeze-dried. These purified fractions from both PDHPC-[α-^{13}C] and PDHPC-cont. were coded as PDHPC-IS-[α-^{13}C] and PDHPC-IS-cont., respectively. The isolation of water-soluble PDHPC was carried out by the method of adsorption chromatography on Toyopearl HW-40 S (4.0×45 cm) reported by Watanabe *et al.*[23] Namely, water-soluble fractions were applied to a Toyopearl HW-40 S column, and then the water-soluble sugar and DHP were thoroughly eluted with water from the column. The sugar content of the eluted solution was determined by UV at 485 nm, after the treatment, by the phenol-sulfuric acid method.[24] The DHP content was determined by UV-spectrophotometric method at 280 nm.[25] Thereafter, the eluent was changed to 50% aqueous dioxane to recover water-soluble PDHPC. To determine the contents of sugar and DHP, the same method was used. The products from both PDHPC-[α-^{13}C] and PDHPC-cont. were coded as PDHPC-WS-[α-^{13}C] and PDHPC-WS-cont., respectively.

^{13}C-NMR determination

The samples were dissolved in DMSO-d_6 (0.5 mL in 5 mm tube). ^{13}C-NMR spectra were obtained on a BRUKER AVANCE 600 Superconducting UltraShieldTM Fourier-Transform NMR Spectrometer (600 MHz, Bruker Corporation, Switzerland), which was equipped with a 5 mm broad-band probe tuned to 150.9 MHz, at 50 °C with a scanning width of 0-220 ppm. The acquisition time of 0.9 s and a relaxation delay of 1.75 s were used. Both the scan of 3000 for PDHPC-WS and the scan of 10000 for PDHPC-IS were accumulated. For purposes of comparison, the spectra were normalized on the basis of the resonance at 56 ppm, which is assigned to the carbon of OCH_3 group.

RESULTS AND DISCUSSION

Isolation of PDHPC-WS by adsorption chromatography

As shown in Fig. 2, the water-soluble fractions from the enzymatic degradation of PDHPC have absorptions at 280 nm and 485 nm. The band at 280 nm indicated a typical absorption of the aromatic ring of DHP. Meanwhile, the band at 485 nm showed the absorption of sugar treated by phenol-H_2SO_4. The solution of water-soluble degraded pectin, free DHP and residual enzymes was eluted out in the first stage of separation with water as mobile phase. In the last stage of separation, the eluent was changed to 50% aqueous dioxane to recover water-soluble PDHPC, containing both phenyl and sugar structures, because it had absorptions at 280 nm and 485 nm, respectively. This result indicated that pectin-DHP complexes could be formed between pectin and DHP during the lignification of coniferin.

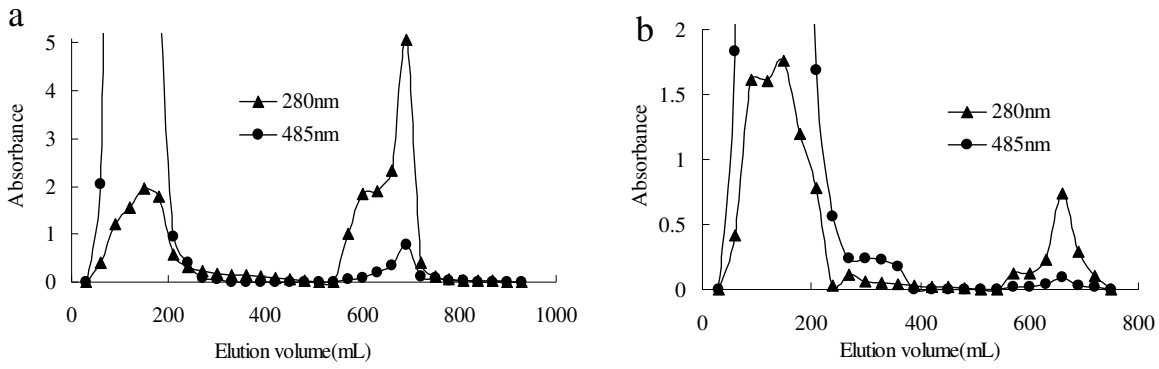

Figure 2: Adsorption chromatogram profile of enzymatic hydrolyzates from PDHPC-cont (a) and PDHPC-[α-^{13}C](b)

Characterization of PDHPC-WS

The ^{13}C-NMR spectra of PDHPC-WS-cont. and PDHPC-WS-[α-^{13}C] are shown in Fig. 3. The signals are assigned based on the reports of Lüdemann *et al*.[26] and Xie *et al*.,[14,19] as shown in Table 1. The guaiacyl-type DHP substructures and the main linkages of the pectin-DHP complex are shown in Fig. 4. By comparingd the spectrum of PDHPC-WS-[α-^{13}C] with that of PDHPC-WS-cont., it was found that the intensity of signal at 191 ppm (No. 2, Fig. 3) was enhanced when labeled coniferin was used. Combining the assignments of signals, this signal was assigned to α-aldehyhyde group (8, Fig. 4). Signal No. 5 at 154.3 ppm assigned to C-α in coniferyl aldehyde (2, Fig. 4) was greatly enhanced by the α-^{13}C enrichment. It was concluded from Fig. 3 that the intensities of signal No. 13 (88.3 ppm, Fig. 3) and signal No. 15 (85.3 ppm, Fig. 3) were significantly enhanced due to α-^{13}C enrichment. The mentioned signals were related to the C-α in β-5 (3, Fig. 4) and β-β (4, Fig. 4) substructures, respectively. An enhanced signal also occurred at 76.1 ppm (No. 17, Fig. 3), which was assigned to the α-carbon of the DHP side chain with ester bond to pectin, as shown in Fig. 4 (6). In addition, the signal of C-α in the β-1 structure (5, Fig. 4) was also enhanced due to α-^{13}C enrichment, which was assigned at 62.9 ppm (No. 23, Fig. 3). As a result of the NMR assignments, it was concluded that DHP can connect to pectin through ester bonds at α position of the side chain during coniferin dehydrogenation. The PDHPC-WS fraction mainly contains β-5, β-β, β-1 and coniferyl aldehyde substructures.

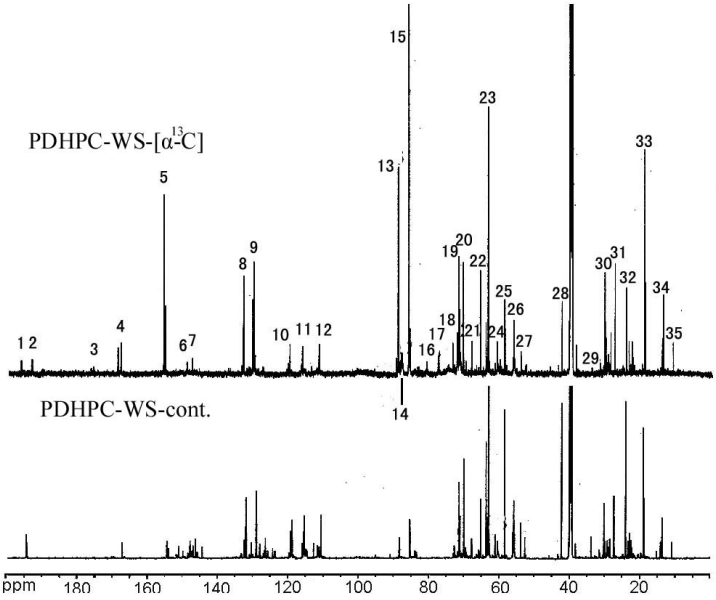

Figure 3: ^{13}C-NMR spectra of PDHPC-WS

Table 1
Chemical shifts and assignments of major signals in PDHPC-WS

Signal No.	Chemical shifts (δ, ppm)		Assignments
	PDHPC-WS-cont.	PDHPC-WS-[α-^{13}C]	
1	194.0	194.2	α-CO in β-O-4, γ-CHO in cinnamaldehyde
2	—	191.2	α-CHO
3	—	174.2	C=O in aliphatic acid
4	167.1	167.1	C=O in aromatic acid
5	154.3	154.2	C-α in coniferyl aldehyde
6	150-152	150-152	C3/C4 in G
7	143-147	143-147	C3/C5 in G
8	131.7	131.5	C1 in G

9	128.8	128.7	Cα in coniferyl alcohol
10	124-126	124-126	C6 in G etherified with α C=O
11	118.7-119.1	118.4-119.2	C6 in G
12	115.4	115.3	C5 in G
13	88.3	88.3	Cα(β-5)
14	—	87.3	Cα(β-5, β-β), Cβ(β-O-4)
15	85.3	85.3	Cα (β-β)
16	—	80.0	Cβ in β-O-4
17	—	76.1	Cα esterified to pectin
18	72.6	72.6	Cα in β-O-4
19	71.3	71.5	Cα in β-O-4, C5 in α-galactose
20	69.9	69.8	C3,4 in α-galactose
21	66.8	67.5	C2 in α-galactose
22	65.2	65.1	Unknown
23	62.9	62.9	Cα/Cβ in β-1, Cγ in β-5
24	61	61	Cγ in β-O-4
25	58.4	58.3	Unknown
26	55.7	55.7	—OCH_3
27	53.7	53.7	Cβ in β-β and β-5
28	42.2	42.2	α-CH_2- at side chain of phenylpropane units
29	32.0	32.1	α-CH_2- at side chain of phenylpropane units
30	30	31	β-CH_2- at side chain of phenylpropane units
31	27	26.8	-CH_2 in saturated alkyl
32	24	24.1	-CH_2 in saturated alkyl
33	18.8-19.0	18.8	Cα in -CH_3
34	13.7	13.7	-CH_3 in saturated alkyl
35	11	11	-CH_3 in saturated alkyl

G. – guaiacyl

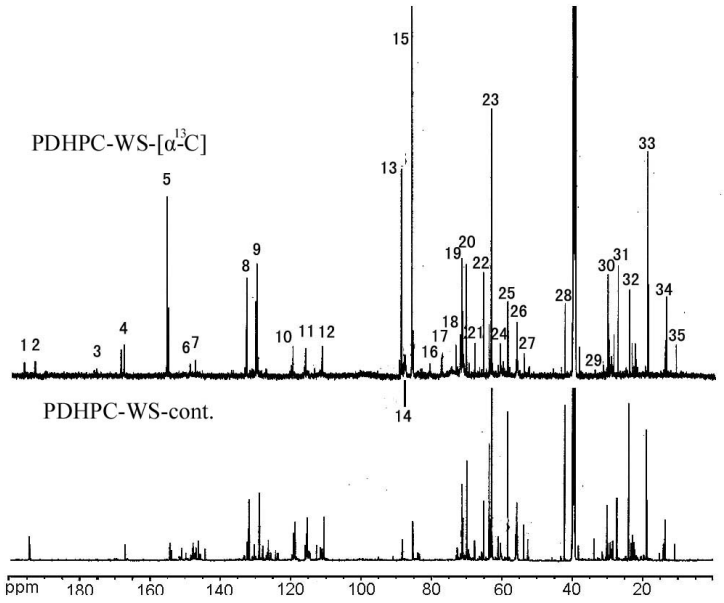

Figure 3: ^{13}C-NMR spectra of PDHPC-WS

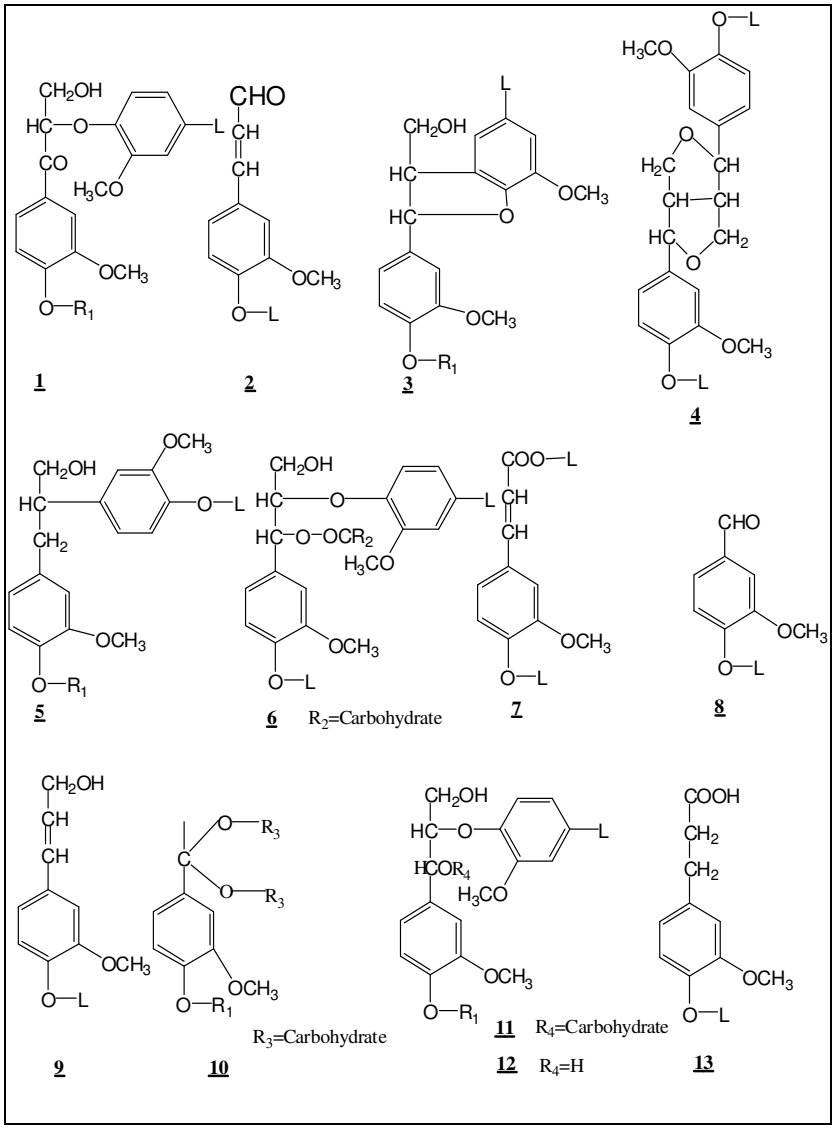

Figure 4: Guaiacyl-type DHP substructures (R_1=H, alkyl or aryl, L=Polylignol)

Table 2
Chemical shifts and assignments of major signals in PDHPC-IS

Signal No.	Chemical shifts (δ, ppm)		Assignments
	PDHPC-IS-cont.	PDHPC- IS-[α-^{13}C]	
1	194.0	194.1	α-CO in β-O-4, γ-CHO in cinnamaldehyde
2	191.0	191.0	α-CHO
3	175.1	175.0	-COOH in aliphatic acid
4	172.3	172.1	-COO- in aliphatic esters or acetyl
5	169.6	169.5	C=O in acetyl of second carbon
6	154.0	154.1	C-α in coniferyl aldehyde
7	150.0	150.1	C3/C4 in G
8	147.2	147.3	C3/C5 in G
9	144.0	144.2	C4/C4' in etherified 5-5'
10	135.0	135.2	C1 in G etherified
11	130	129.7	Cα in coniferyl alcohol
12	126.5	126.3	C6 in G etherified with α C=O
13	119.2	119.2	C6 in G

14	115.2	115.2	C5 in G
15	111.3	111.2	C2 in G
16	101.5	101.2	C1/C5 in G
17	—	100.0	Cα with acetal linkage
18	87.1	87.2	Cα in β-5
19	85.1	85.2	Cα in β-β
20	84.0	84.2	Cα etherified to pectin, Cβ in β-O-4
21	81-83	81-83	Cβ in β-O-4
22	74.2	74.3	Cα esterified to pectin
23	71.8	71.6	Cα in β-O-4
24	63.2	63.2	Cγ in β-5, β-1 and coniferyl alcohol
25	61.8	61.7	Cγ in β-O-4, C6 in α-galactose
26	60.1	60.3	
27	55.9	55.9	-OCH_3
28	53.9	53.8	Cβ in β-β and β-5
29	32.0	32.1	α-CH_2- at side chain of phenylpropane units
30	29.2	29.1	β-CH_2- at side chain of phenylpropane units
31	21	21	-CH_3 of acetyl
32	14	14	-CH_3 in saturated alkyl
33	11	11	-CH_3 in saturated alkyl

G. – guaiacyl

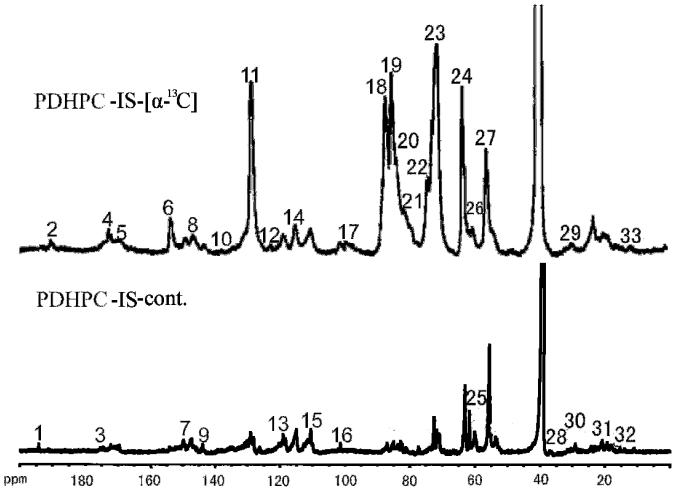

Figure 5: ^{13}C-NMR spectra of PDHPC-IS

Characterization of PDHPC-IS

As shown in Fig. 5, the intensity of signal No. 2 at 191 ppm in the spectrum of PDHPC-IS-[α-^{13}C] is enhanced by the α-^{13}C-enrichment and can be assigned to C-α of vanillin (8, Fig. 4) type subunits. Signal No. 6 at 154.1 ppm can not be observed in the spectrum of PDHPC-IS-cont. However, this signal in the spectrum of PDHPC-IS-[α-^{13}C] is enhanced to some extent, which is assigned to the C-α in coniferyl aldehyde (2, Fig. 4). A remarkably enhanced ^{13}C resonance is also noted at 129.7 ppm (No. 11), which is assigned to the Cα in coniferyl alcohol (9, Fig. 4). Signal No. 17 at 100.0 ppm is enhanced by α-^{13}C enrichment. This signal is assigned to α-carbon of the DHP side chain with acetal linkage to pectin, as shown in Fig. 4 (10), according to the ^{13}C-NMR spectra of model compound.[27] The enhanced signals from 85.2 ppm to 87.2 ppm can be easily assigned to Cα in β-5 (3, Fig. 4) and β-β (4, Fig. 4). The intensity of signal No. 20 at 84.2 ppm is enhanced, which is assigned to α-C in benzyl ether linkages to pectin (11, Fig. 4)[14,19,28] and β-C in α-CO containing β-O-4 (1, Fig. 4). As a result of α-^{13}C enrichment, signal No. 22 at 74.3 ppm arisen from Cα, which was esterified to pectin, was enhanced (6, Fig. 4).[14,19,29] It indicates that DHP is linked with pectin through an ester bond. Another signal at 71.6 ppm (No. 23) is assigned to α-C of β-O-4 structure. In the spectrum of PDHPC- IS-[α-^{13}C], a weak enhanced signal at

32.1 ppm (No. 29) can be observed. This evidenced the existence of saturated α-methylene group in phenylpropane unit (13, Fig. 4).

CONCLUSION

The pectin-DHP complex can be prepared from either unlabelled or labeled coniferin in the presence of pectin by horseradish peroxidase, β-glucosidase and glucose oxidase. NMR results clearly indicated that DHP could associate with pectin through a benzyl ether linkage, ester bonds and acetal linkage. Three linkages occurred at α-position of phenylpropane units. It was also found that the main substructures in the DHP were β-O-4, β-β, β-5, β-1, coniferyl alcohol and coniferyl aldehyde structures. There are also substructures in the DHP segments, such as vanillin, phenylpropane units with α-methylene substructures.

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