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Methylation and 2D NMR analysis of arabinoxylan from the seeds of *Plantago asiatica* L.

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ABSTRACT

PLP-3 (*Plantago asiatica* L. polysaccharide 3) was isolated and purified from the seeds of *Plantago asiatica* L. Its structure characters were elucidated by monosaccharide composition analysis, partial acid hydrolysis and methylation analysis, combined with FT-IR, GC/MS, 1D and 2D NMR spectroscopy. PLP-3 was found to be arabinoxylan, containing glucuronic acid. It was consisted of β-1,4-linked Xyl*p* backbone with short side chains attached to its *O*-2 (1,2,4-linked Xyl*p*, 17.87%) or *O*-3 (1,3,4-linked Xyl*p*, 24.24%) positions. The main terminal residues were T-linked Ara*f* (8.13%), T-linked Xyl*p* (15.58%) and T-linked GlcA*p* (13.22%). Small amounts of other residues were also found in PLP-3, such as 1,2,5-linked Ara*f*, 1,2-linked Rha*p*, T-linked Glc*p* and T-linked Gal*p*. A possible molecular structure was proposed.

1. Introduction

Plantago asiatica L. is a species of *Plantago* family, widely distributed in China, especially in Jiangxi Province. The seeds of *P. asiatica* L. were considered as orthodox product in ancient Chinese. Recent studies found that polysaccharide isolated and/or purified from the seeds of *P. asiatica* L. induced the maturation of murine DCs (Huang, Tang, et al., 2009; Huang, Xie, et al., 2009) and exhibited antioxidant activities (Yin, Nie, Zhou, Wan, & Xie, 2009).

In previous report, crude polysaccharide isolated from the seeds of *P. asiatica* L. was purified into PLP-1 (18.9%), PLP-2 (52.6%) and PLP-3 (28.5%). PLP-2 was high in sugar content (87.32%) and low in protein content (1.16%). Its optical rotation was −6.2. The structure feature of PLP-2 was revealed as a highly branched heteroxylan consisted of β-1,4-linked Xyl*p* backbone with side chains attached to *O*-2 (1,2,4-linked Xyl*p*, 10.5%) or *O*-3 (1,3,4-linked Xyl*p*, 24.9%) positions. The side chains consisted of β-T-linked Xyl*p*, α-T-linked Ara*f*, α-T-linked GlcA*p*, β-Xyl*p*-(1→3)-α-Ara*f* and α-Ara*f*-(1→3)-β-Xyl*p* (Yin et al., 2011).

During the structural characterization process, PLP-1 and PLP-3 were found to have similar structural features. The objective of current study was to elucidate the structure of PLP-3. It was elucidated using partially acid hydrolysis, methylation analysis, GC/MS, 1D and 2D NMR spectroscopy, including homonuclear $^{1}H/^{1}H$ correlation spectroscopy (DQF-COSY and TOCSY) and heteronuclear $^{13}C/^{1}H$ multiple-quantum correlation experiments (HSQC and HMBC).

2. Experimental

2.1. Materials

The seeds of *P. asiatica* L. were purchased from Ji'an County (Jiangxi Province, China) and dried before used. The species were identified by Dr. Cui-sheng Fan, Jiangxi University of Traditional Chinese Medical (Nanchang, China). PLP-3 was isolated and purified from the seeds of *P. asiatica* L. as described in previous report (Yin et al., 2011). The contents of sugar, protein and uronic acid in PLP-3 were 79.28, 0.72 and 20.13%, respectively.

Monosaccharide standards of mannose (Man), rhamnose (Rha), ribose (Rib), galactose (Gal), xylose (Xyl), arabinose (Ara), fucose (Fuc) and glucose (Glc) were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Hydroxylamine hydrochloride, pyridine, trifluoroacetic acid (TFA), chloroform and sulfuric acid were of analytical pure grade, and purchased from Shanghai Chemical Reagent Co. (Shanghai, China). Aqueous solutions were prepared with ultra-pure water from a Milli-Q water purification system (Millipore, Bedford, MA, USA). All other chemicals and reagents were of analytical grade.

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2.2. Monosaccharide composition analysis

The sample was hydrolyzed by 2 M TFA at 100 °C for 12 h and the hydrolysate was acetylated according literature (Chen, Xie, Nie, Li, & Wang, 2008). Alditol acetates of authentic standards and sample were prepared and subjected for gas chromatography (GC) analysis. GC was equipped with a DB-1701 capillary column (30 m × 0.25 mm × 0.25 μm) and a flame ionization detector. An initial column temperature was held at 170 °C for 2 min, then programmed at a rate of 10 °C/min to 250 °C and held at 250 °C for 10 min.

2.3. Methylation and GC–MS analysis

The reduction of uronic acid was conducted following a procedure described by (Taylor & Conrad, 1972) with slight modifications. PLP-3 was added into distilled water and reduced with 1-cyclohexyl-3-(2-morpholinoethyl)-carbodiimide methyl-p-toluenesulfonate (CMC, Sigma). The reduced polysaccharide (PLP-3-R) was subjected for methylation analysis.

Methylation analysis of polysaccharide (PLP-3 and PLP-3-R) was conducted according to the method of previous reports (Ciucanu & Kerek, 1984; Nie et al., 2011) with some modifications. The methylated polysaccharide was then converted into partially methylated alditol acetates (PMAA) by hydrolysis, reduction with $NaBD_4$, and acetylation, followed by linkage analysis using an Agilent 7890-7000A GC–MS system with a SP-2330 column (Supelco, Bellefonte, PA; 30 m × 0.25 mm, 0.2 μm film thickness). The oven conditions included as initial temperature of 160 °C, 2 °C/min to 210 °C, and finally 5 °C/min to 240 °C. The individual peaks of the PMAA and fragmentation patterns were identified by their mass spectra and relative retention time in GC (Biermann & McGinnis, 1989). The percentage of methylated sugars was estimated as ratios of the peak areas (total ion current).

2.4. Partial acid hydrolysis

PLP-3 (60 mg) was hydrolyzed with 0.03 M TFA at 90 °C for 1 h. After cooling, TFA was evaporated with methanol under reduced pressure. The hydrolysate was dissolved and dialyzed against distilled water to obtain higher molecular weight fraction (PLP-3-P-H, 40.5 mg). PLP-3-P-H was collected for methylation and NMR analysis.

2.5. UV and FT-IR spectrum

Ultraviolet–visible spectrum was recorded with a TU-1900 Ultraviolet-Visible Spectrophotometer (PGENERAL, Beijing, China). FT-IR was recorded with a Thermo Nicolet 5700 infrared spectrometer (Thermo Electron, Madison, WI, USA) at the absorbance mode from 4000 cm^{-1} to 400 cm^{-1} and a resolution of 4 cm^{-1}, using KBr disks method.

2.6. NMR spectrum

PLP-3-P-H (30 mg) was exchanged with 99.9% of D_2O for thrice and dissolved in 0.5 ml D_2O for 6 h before NMR analysis. The 1D and 2D NMR spectrum of sample were recorded on a Bruker DRX-600 NMR spectrometer (Bruker, Rheinstetten, Germany). The spectra of ^1H, ^{13}C, DQF-COSY, TOCSY, HSQC and HMBC experiments were conducted at 293 K. The ^1H NMR spectrum was recorded by fixing HOD signal at δ 4.80 ppm. The ^{13}C NMR spectrum was recorded using acetone (HPLC grade) as an internal standard, fixing the methyl carbon signal at δ 30.24 ppm.

3. Results and discussion

3.1. Monosaccharide composition

The monosaccharide composition of PLP-3 was analyzed by GC. Compared with monosaccharide standards, PLP-3 was composed of Rha (1.85%), Ara (39.56%), Xyl (55.93%), Glc (0.83%) and Gal (1.83%). It indicated PLP-3 was arabinoxylan. Monosaccharide composition of PLP-3 was similar to that of PLP-2, but the ratio of Ara and Xyl was a little higher (Yin et al., 2011). Compared with PLP-2, the compositional results of PLP-3 suggested the two fractions may have different structural characteristics.

3.2. Methylation and GC–MS analysis

The methylation analysis results are presented in Table 1. PLP-3 was highly branched for the existing of 1.03% of 1,2,5-linked Ara*f*, 17.87% of 1,2,4-linked Xyl*p* and 24.24% of 1,3,4-linked Xyl*p*. The results confirmed Xyl was present only in pyranose ring form which was similar to PLP-2 (Yin et al., 2011) and other reports (Fischer et al., 2004; Guo, Cui, Wang, & Christopher Young, 2008). It should be noted that trace of Ara in PLP-3 was detected in pyranose ring (T-linked Ara*p*).

According to literatures (Fischer et al., 2004; Guo et al., 2008; Samuelsen, Cohen, Paulsen, Brull, & Thomas-Oates, 1999; Yin et al., 2011), 1,2,4-linked Xyl*p* and 1,3,4-linked Xyl*p* residues was assumed in the backbone. Mainly non-reducing ends in PLP-3 were T-linked Xyl*p* (8.13%) and T-linked Ara*f* (15.58%). The high proportion of 1,3-linked Ara*f* (16.82%) and 1,3-linked Xyl*p* (7.47%) indicated both of them were probably in branch areas. There were small amounts of 1,2-linked Rha*p*, 1,3-linked Rha*p*, T-linked Gal*p*, T-linked Glc*p*, 1,2-linked Glc*p* and 1,3-linked Glc*p*. Unlike PLP-2 (Yin et al., 2011), neither 1,3,4-linked Gal*p* nor 1,3,6-linked Glc*p* residues were found in PLP-3.

Methylation analysis of reduced PLP-3 (PLP-3-R) showed a great increase of T-linked Glc*p* (from 0.34% to 13.22%). It suggested that uronic acid in PLP-3 was presented as T-linked GlcA*p*.

3.3. Partial acid hydrolysis

PLP-3 was hydrolyzed with 0.03 M TFA at 90 °C to get a higher molecular weight fraction PLP-3-P-H. GPC result showed PLP-3-P-H was homogeneous with an average molecular weight of 562 kDa (not shown). Methylation analysis of PLP-3-P-H is shown in Table 1. T-linked Ara*f* (5.45%), 1,3-linked Ara*f* (13.55%), T-linked Xyl*p* (12.41%), 1,3-linked Xyl*p* (9.48%), 1,4-linked Xyl*p* (13.92%), 1,2,4-linked Xyl*p* (20.76%) and 1,3,4-linked Xyl*p* (21.04%) were major residues. Compared with PLP-3, only T-linked Ara*f*, T-linked Xyl*p* and 1,3-linked Ara*f* reduced slightly, while 1,4-linked Xyl*p* increased greatly. It suggested some T-linked Ara*f*, T-linked Xyl*p* and 1,3-linked Ara*f* were probably linked to *O*-2 or *O*-3 positions of 1,4-linked Xyl*p*. The majority branching residues were 1,2,4-linked Xyl*p* and 1,3,4-linked Xyl*p*. It re-confirmed that PLP-3 was highly branched.

3.4. UV and FT-IR spectrum

The UV spectrum of the PLP-3 shows no obvious absorption at 254 and 280 nm which indicated little protein in PLP-3 (not shown).

The FT-IR spectrum of PLP-3 and PLP-3-P-H is shown in supplementary data. The bands in the region of 3384.3 cm^{-1} were due to the hydroxyl stretching vibration of PLP-3. The bands in the region of 2927.7 cm^{-1} were due to C—H stretching vibration. Besides, absorption band at 1725.5 cm^{-1} was assigned to the group of C═O in uronic acid in PLP-3. There was a strong band between 900 and 1200 cm^{-1} attributed to the stretching vibrations of

Table 1
Glycosyl-linkage compositions of PLP-3, PLP-3-R and PLP-3-P-H.

Corresponding derivatives	Residue linkage	PLP-3 (%)[a]	PLP-3-R (%)[a]	PLP-3-P-H (%)[a]
2,3,5-Me_3-1,4-Ac_2-Arabinitol	T-linked Ara*f*	8.13	7.29	5.45
2,3,4-Me_3-1,5-Ac_2-Arabinitol	T-linked Ara*p*	0.49	0.21	0.14
2,5-Me_2-1,3,4-Ac_3-Arabinitol	1,3-linked Ara*f*	16.82	14.97	13.55
3-Me-1,2,4,5-Ac_4-Arabinitol	1,2,5-linked Ara*f*	1.03	0.43	0.64
2,3,4-Me_3-1,5-Ac_2-Xylitol	T-linked Xyl*p*	15.58	12.66	12.41
2,4-Me_2-1,3,5-Ac_3-Xylitol	1,3-linked Xyl*p*	7.47	7.17	9.48
2,3-Me_2-1,4,5-Ac_3-Xylitol	1,4-linked Xyl*p*	5.24	5.22	13.92
3-Me-1,2,4,5-Ac_4-Xylitol	1,2,4-linked Xyl*p*	17.87	13.57	20.76
2-Me-1,3,4,5-Ac_4-Xylitol	1,3,4-linked Xyl*p*	24.24	21.36	21.04
3,4-Me_2-1,2,5-Ac_3-Rhamnitol	1,2-linked Rha*p*	1.07	2.07	1.19
2,4-Me_2-1,3,5-Ac_3-Rhamnitol	1,3-linked Rha*p*	0.02	–	0.25
2,3,4,6-Me_4-1,5-Ac_2-Glucitol	T-linked Glc*p*	0.34	13.22	0.37
3,4,6-Me_3-1,2,5-Ac_3-Glucitol	1,2-linked Glc*p*	0.04	0.61	0.32
2,3,4,6-Me_4-1,5-Ac_2-Galactitol	T-linked Gal*p*	1.09	0.91	0.53

'–' not determined.

[a] Calculated a percentage of all partially methylated alditol acetates present, based on the peak area.

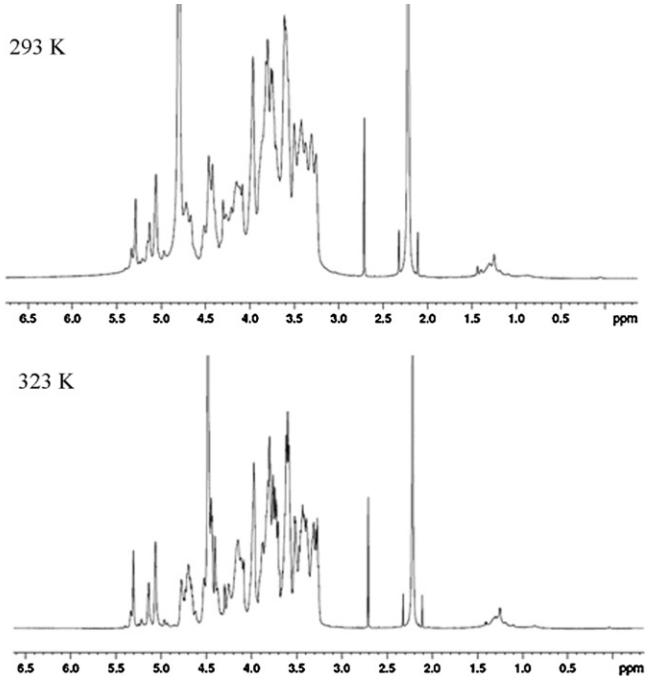

Fig. 1. ^1H NMR (600.1 MHz) spectrum of PLP-3-P-H determined at 293 K and 323 K.

pyranose ring which was in accordance with methylation result. A characteristic absorption at 902.4 cm^{-1} was observed, indicating the β-configuration of the sugar units (Barker et al., 1954). The absorption at 833.6 cm^{-1} was determined which was a characteristic absorption of α-configuration. The characteristic absorption at 802.2 cm^{-1} corresponding to the existence of Man (Mathlouthi & Koenig, 1987) was not determined. The FT-IR spectra suggested PLP-3 was polysaccharide which contained uronic acid. The spectrum of PLP-3-P-H was similar to that of PLP-3.

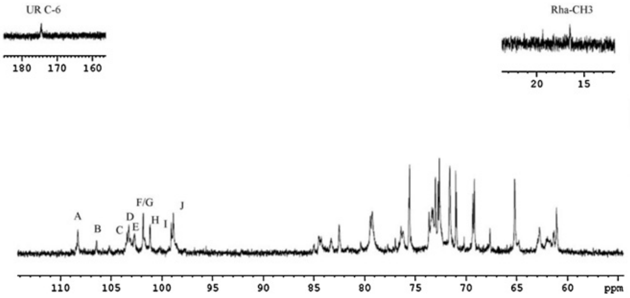

Fig. 2. ^{13}C NMR (151.0 MHz) spectrum of PLP-3-P-H.

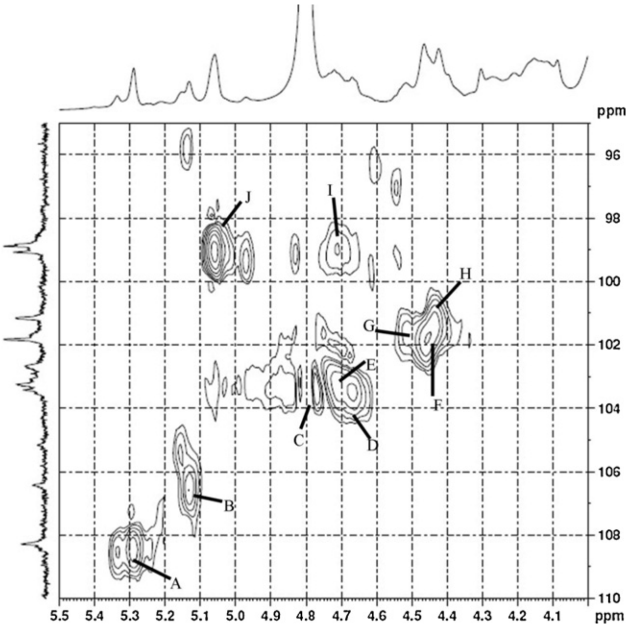

Fig. 3. Part of HSQC spectrum of PLP-3-P-H.

3.5. NMR spectrum

PLP-3 was too viscous to get a high concentration in D_2O. The above results showed that PLP-3-P-H retained native polysaccharide PLP-3's structural information well. Therefore, PLP-3-P-H was applied for 1D and 2D NMR analysis to get more meaningful information to elucidate PLP-3's structure.

Fig. 1 shows the ^1H NMR spectrum of PLP-3-P-H. The spectrum determined at 323 K was aimed to obtain proton signals distributed in the area of δ 4.60–4.90 ppm. The region of δ 5.1–5.4 ppm and δ 4.4–4.8 ppm were considered to be zone of accumulation of α-linked Ara*f* and β-linked Xyl*p*, respectively (Apirattananusorn, Tongta, Cui, & Wang, 2008; Ebringerova, Hromadkova, & Berth, 1994; Hoffmann, Kamerling, & Vliegenthart, 1992). In its ^{13}C NMR spectrum (Fig. 2), the signal at δ 16.49 ppm could be assigned to the methyl carbon of Rha. The ^{13}C NMR spectrum of PLP-3-P-H shows nine anomeric carbon signals at δ 108.28, 106.24, 103.42, 103.24, 102.67, 101.82, 101.15, 99.07 and 98.88 ppm, which were labeled as A, B, . . ., K and I according to their decreasing anomeric carbon

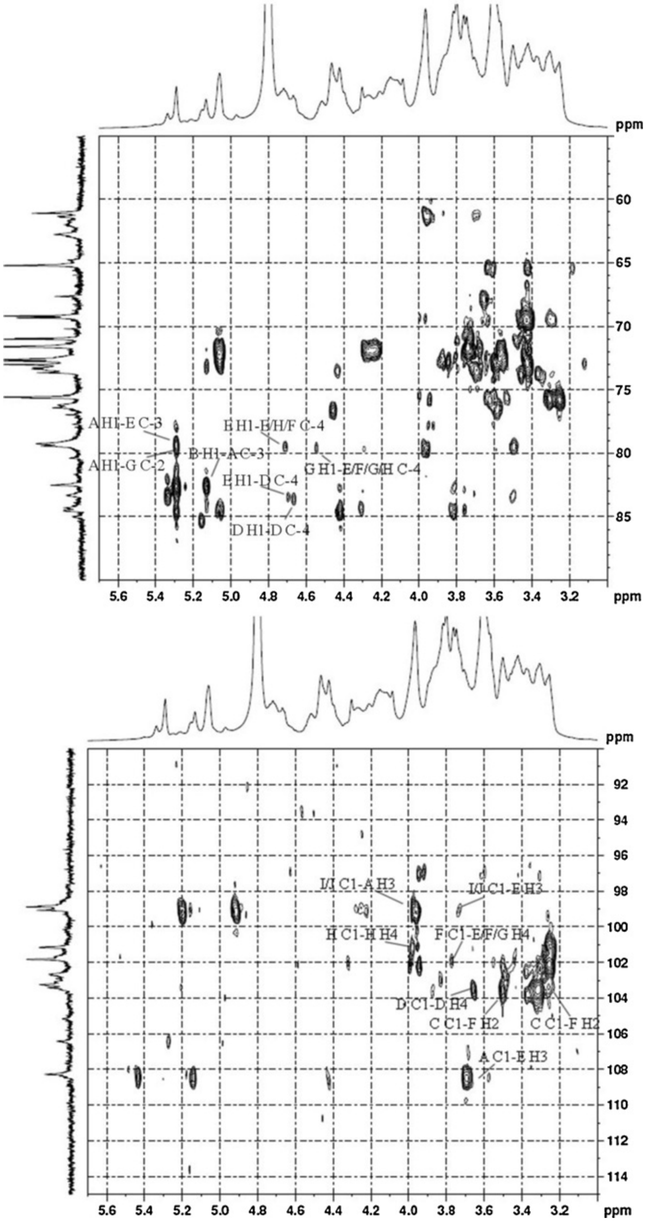

Fig. 4. Part of HMBC spectrum of PLP-3-P-H.

signals. Their corresponding anomeric proton signals in ^{1}H NMR were labeled with the contribution of HSQC. All ^{1}H and ^{13}C NMR were assigned from DQF-COSY, TOCSY, HSQC (Fig. 3) and HMBC (Fig. 4) spectrum. The main residues' chemical shifts of PLP-3-P-H were assigned completely (Table 2).

Residue A had anomeric carbon chemical shift at δ 108.28 ppm. Methylation result shows that the amount of 1,3-linked Ara*f* was much more than T-linked Ara*f*. It suggested residue A was 1,3-linked Ara*f*. Its anomeric proton chemical shift was at δ 5.29 ppm which indicated it was α-linked. Other proton and carbon chemical shifts were assigned completely and in agreement with literature values (Fischer et al., 2004; Kang et al., 2011).

For residue B, the H-1 signal at δ 5.13 ppm indicated it was α-linked. Its anomeric carbon chemical shift was at δ 106.42 ppm. The intensity was much weaker than residue A. That suggested it was T-linked Ara*f*. Other proton chemical shifts were assigned from COSY spectrum and confirmed by DQF-COSY spectrum. The carbon signals of residue B were observed at δ 79.44, 76.98, 84.97 and 61.32 ppm corresponding to C-2, C-3, C-4 and C-5 from HSQC spectrum, respectively. They were in consistent with previous information (Das et al., 2009; Habibi, Mahrouz, & Vignon, 2005; Kang et al., 2011).

Residue C had anomeric carbon and proton chemical shift at δ 103.42 and 4.77 ppm (from its ^{1}H NMR spectrum determined at 323 K, Fig. 2), respectively. Its anomeric proton chemical shift indicated it was β-linked. According to methylation data and its anomeric carbon intensity, residue C was assigned as 1,3-linked Xyl*p*. The chemical shift of H-2 was obtained from COSY spectrum for its correlation with H-1. H-2, H-3, H-4, H-5 and H-5′ were assigned in the same procedure and confirmed by DQF-COSY spectrum. Its carbon chemical shifts were assigned completely from HSQC spectrum. The downfield shift of C-3 (δ 79.44 ppm) indicated it was 1,3-linked. Its chemical shifts were supported by other reports (Fischer et al., 2004; Rantanen et al., 2007; Samuelsen, Lund, et al., 1999; Yin et al., 2011).

Residue D and E had anomeric carbon chemical shifts at δ 103.24 and 102.67 ppm, with anomeric proton chemical shifts at δ 4.67 and 4.72 ppm respectively. That indicated both of them were β-linked. Methylation results showed 1,3,4-linked Xyl*p* takes amount of 21.04% of total residues. Compared with their anomeric carbon and proton intensity, residue D and E were assigned as β-1,3,4-linked Xyl*p*. The different chemical shifts indicated they were in different chemical environment. The other proton and carbon chemical shifts were assigned completely, both from HSQC, COSY and DQF-COSY spectrum and literature data (Apirattananusorn et al., 2008; Hoije et al., 2006; Yin et al., 2011). The C-3 signal of residue D (δ 76.41 ppm) and E (δ 79.24 ppm) showed downfield shifts which confirmed they were 1,3,4-linked.

Residues F and G were assigned as 1,2,4-linked Xyl*p* as it had a most intensive anomeric carbon chemical at δ 101.82 ppm. Its anomeric proton chemical shifts were at δ 4.47 (for F) and 4.52 ppm (for G) which suggested it was β-linked. The downfield shifts of C-2 (δ 76.22 and 79.44 ppm) indicated the two residues were 1,2,4-linked. The other proton and carbon chemical shifts were supported by literature values (Bendahou, Dufresne, Kaddami, & Habibi, 2007; Fischer et al., 2004; Yin et al., 2011).

According to methylation analysis results, the contents of 1,4-linked Xyl*p* and 1,3-linked Xyl*p* in PLP-3-P-H were 13.92% and 12.41%, respectively. The intensity of anomeric carbon signal of residue H had almost equaled to residue I. The complete carbon signal assignment of residue H showed a similar chemical shifts with that of 1,4-linked Xyl*p* from literature (Bock, Pedersen, & Pedersen, 1984; Du et al., 2009; Hoffmann et al., 1992; Yin et al., 2011). The anomeric proton signal of residue I at δ 4.70 ppm indicated it was β-linked. And the assignment of residue H's signal showed it was β-T-linked Xyl*p*, which was confirmed by literature values (Bock et al., 1984; Fischer et al., 2004; Hoije et al., 2006).

Residue J was assigned as T-GlcA*p* as this spin system consisted of only five protons, with a high chemical shift of H-5 (δ 4.21 ppm). The anomeric signal of residue J at δ 5.06 ppm indicated it was α-linked. The carbon signals of residue J were observed at δ 71.02, 72.05, 76.98, 71.60 and 174.43 ppm for C-2, C-3, C-4, C-5 and C-6, respectively. All the ^{1}H and ^{13}C chemical shifts assigned to residue J in the current paper were consistent with previous reports (Dobruchowska, Gerwig, Babuchowski, & Kamerling, 2008; León de Pinto et al., 2000; Marvelys, Maritza, Lilian, de Pinto Gladys, & Julio, 2006; Yin et al., 2011). NMR assignment of residue J confirmed the FT-IR analysis results which showed there was uronic acid in PLP-3-P-H. Residue J was not methyl-esterified for no signal determined around δ 53 ppm (Samuelsen, Lund, et al., 1999).

The sequence of glycosyl residues in PLP-3-P-H was determined on the basis of a long-range HMBC spectrum (Fig. 4) and summarized in Table 3. The cross peaks of both anomeric protons and carbons of each glycosyl residues of PLP-3-P-H were examined, and both inter- and intra-residual connectivity were observed in Fig. 4.

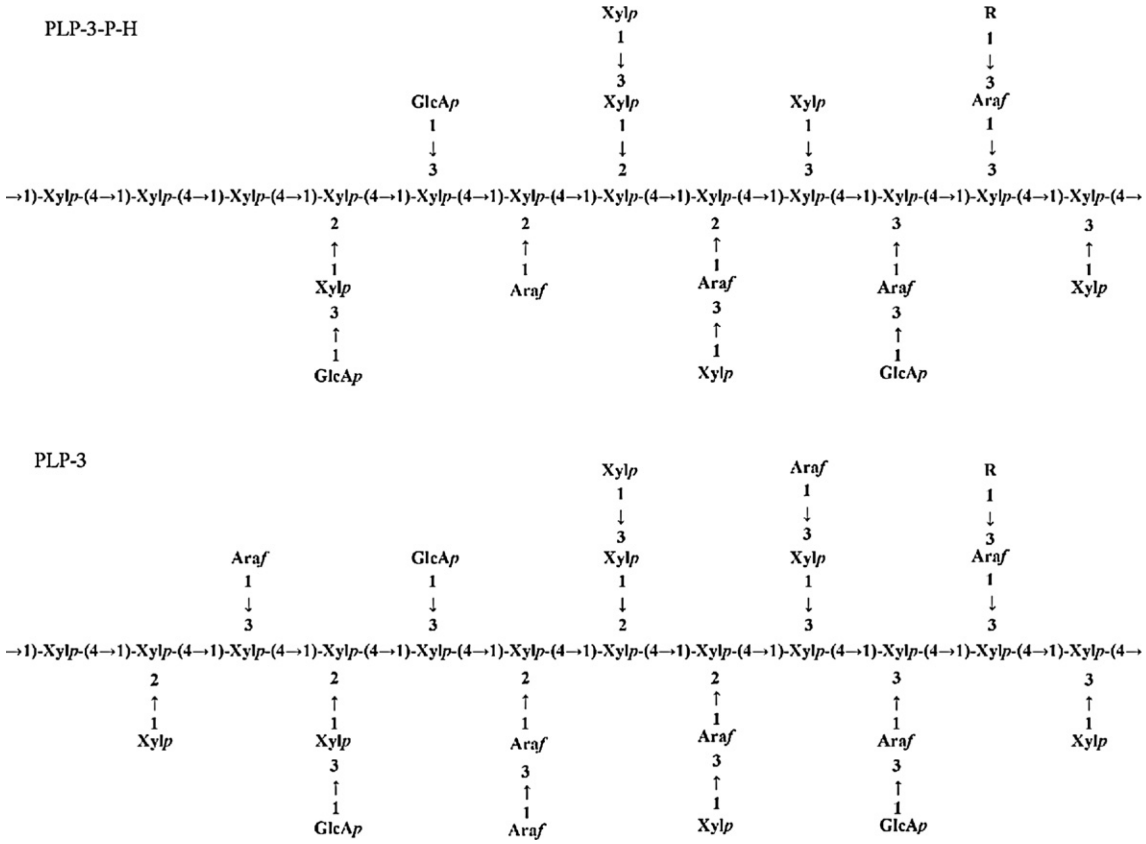

Fig. 5. The proposed structure of PLP-3-P-H and PLP-3. R is one of following residues: 1,2-linked Rha*p*, 1,3-linked Rha*p*, 1,2-linked Glc*p*, T-linked Glc*p*, 1,2,5-linked Ara*f* or T-linked Ara*p*.

Table 2
Chemical shifts assignment of ^{1}H and ^{13}C NMR spectrum of PLP-3-P-H on the basis of HSQC, HMBC, DQF-COSY and TOCSY.

Sugar residues	H-1/C-1	H-2/C-2	H-3/C-3	H-4/C-4	H-5/C-5	H-5′
A	5.29	4.39	3.97	4.20	3.52	3.70
α-1,3-linked Ara*f*	108.28	79.44	82.52	84.97	61.09	
B	5.13	4.30	3.93	4.20	3.64	3.71
α-T-linked Ara*f*	106.42	79.44	76.98	84.97	61.32	
C	4.77	3.38	3.58	3.71	3.85	3.50
β-1,3-linked Xyl*p*	103.42	73.66	79.44	69.33	61.98	
D	4.67	3.32	3.45	3.64	3.78	3.38
β-1,3,4-llinked Xyl*p*	103.24	73.66	76.41	83.31	62.77	
E	4.72	3.30	3.72	3.78	3.93	3.29
β-1,3,4-llinked Xyl*p*	102.67	73.66	79.24	79.44	65.20	
F	4.47	3.26	3.60	3.79	3.98	3.33
β-1,2,4-linked Xyl*p*	101.82	76.22	72.64	79.24	65.02	
G	4.52	3.50	3.82	4.08	3.39	–
β-1,2,4-linked Xyl*p*	101.82	79.44	73.37	80.40	62.77	
H	4.42	3.25	3.43	3.58	3.96	3.30
β-1,4-linked Xyl*p*	101.15	73.02	75.65	79.44	65.20	
I	4.70	3.31	3.47	3.62	3.74	3.35
β-T-linked Xyl*p*	99.07	73.66	76.41	69.18	67.65	
J	5.06	3.61	3.76	3.97	4.21	–
α-T-linked GlcA*p*	98.88	71.02	72.05	76.98	71.60	174.43

‘–’ not determined.

For example, cross-peaks were found between H-1 (δ 5.29 ppm) of residue A and C-2 (δ 79.44 ppm) of residue G, indicating some of β-1,3-linked Ara*f* residues were linked to *O*-2 of β-1,2,4-linked Xyl*p*. Cross-peaks were found between H-1 (δ 5.29 ppm) of residue A and C-3 (δ 79.24 ppm) of residue E, indicating some of β-1,3-linked Ara*f* residues were linked to *O*-3 of β-1,3,4-linked Xyl*p*.

Based on methylation analysis, 1D and 2D NMR spectroscopy, partial acid hydrolysis fraction PLP-3-P-H probable structure was firstly obtained. And PLP-3-P-H's average molecular weight was as much as 562 kDa and it kept well in structure information of PLP-3. Therefore, we could conclude the original polysaccharide's (PLP-3's) probable structure (Fig. 5).

Table 3
The significant connectivities observed in HMBC spectrum for anomeric proton/carbon of the sugar residues of PLP-3-P-H.

Sugar residues	Sugar linkage	H-1/C-1	Observed connectivities		
		δ_H/δ_C	δ_H/δ_C	Residue	Atom
A	1,3-linked Ara*f*	5.29	79.44	G	C-2
			79.24	E	C-3
		108.28	3.72	E	H-3
B	T-linked Ara*f*	5.13	82.52	A	C-3
		106.42			
C	1,3-linked Xyl*p*	4.77			
		103.42	3.26	F	H-2
			3.50	G	H-2
D	1,3,4-linked Xyl*p*	4.67	83.31	D	C-4
		103.24	3.64	D	H-4
E	1,3,4-linked Xyl*p*	4.72	83.31	D	C-4
			79.44	E	C-4
			79.24	H	C-4
			79.24	F	C-4
		102.67			
F	1,2,4-linked Xyl*p*	4.47			
		101.82	3.78	E/F/G	H-4
G	1,2,4-linked Xyl*p*	4.52	79.44/79.24	E/G/H/F	C-4
		101.82			
H	1,4-linked Xyl*p*	4.42			
		101.15	3.95	H	H-4
I	T-linked Xyl*p*	4.70			
		99.07	3.96	A	H-3
			3.72	E	H-3
J	T-linked GlcA*p*	5.06			
		98.88	3.96	A	H-3
			3.72	E	H-3

4. Conclusion

In this study, PLP-3 was characterized to be arabinoxylan. It was highly branched which consisted of a β-1,4-linked Xyl*p* backbone, with short side chains attached to its *O*-2 (β-1,2,4-linked Xyl*p*, 17.87%) or *O*-3 (β-1,3,4-linked Xyl*p*, 24.24%) positions. Its mainly terminal residues were α-T-linked Ara*f*, β-T-linked Xyl*p* and α-T-linked GlcA*p*. It is interesting to find out that some researchers suggested 1,2,3,4-linked Xyl*p* as a single branch point due to the possibility of lone free hydroxyl group escaped in the methylation process (Fischer et al., 2004), while there were many studies (Samuelsen, Lund, et al., 1999; Edwards, Chaplin, Blackwood, & Dettman, 2003; Guo et al., 2008) reported that there was 1,2,3,4-linked Xyl*p* in polysaccharides from *Plantago* family. And arabinoxylans from other sources were also reported to have 1,2,3,4-linked Xyl*p* residues (Edwards et al., 2003; Rao & Muralikrishna, 2007; Sun, Cui, Gu, & Zhang, 2011). Therefore, we assumed that arabinoxlyan in the nature probably had 1,2,3,4-linked Xyl*p* linkage. But our previous (Yin et al., 2011) and present study suggested polysaccharides from the seeds of *Plantago asiatica* L. did not contain this kind linkage. It was probably attributed to area and species difference.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.carbpol.2012.02.025.

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