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Structural characterization of a highly branched polysaccharide from the seeds of *Plantago asiatica* L.

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ABSTRACT

In this paper, polysaccharides were extracted from the seeds of *Plantago asiatica* L. with hot water and separated into three fractions PLP-1 (18.9%), PLP-2 (52.6%) and PLP-3 (28.5%) by Sephacryl™ S-400 HR column chomatography. The main fraction PLP-2's structure was elucidated using oxalic acid hydrolysis, partial acid hydrolysis, methylation, GC, GC–MS, 1D and 2D NMR. PLP-2 was composed of Rha, Ara, Xyl, Man, Glc and Gal, in a molar ratio of 0.05:1.00:1.90:0.05:0.06:0.10. Its uronic acid was GlcA. PLP-2 was highly branched heteroxylan which consisted of a β-1,4-linked Xyl*p* backbone with side chains attached to *O*-2 or *O*-3. 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*, etc. Based on these results, the structure of PLP-2 was proposed.

1. Introduction

Recent years, polysaccharides from *Plantago* family have attracted considerable attention because they have been demonstrated to possess diverse pharmacological activities, such as protecting against pneumococcal infection in mice (Hetland, Samuelsen, & Loslash, 2000), promoting the healthy function of the colon (Alabaster, Tang, & Shivapurkar, 1996) and improving status in mild-moderate hypercholesterolaemic individuals (Solà et al., 2010).

Polysaccharide's biological activities are related to its structure character. Marlett and Fischer found out that a gel-forming fraction of the alkali-extractable polysaccharide of *Plantago ovata* Forsk was responsible for increasing stool output and lowering blood cholesterol levels in humans (Marlett & Fischer, 2002). It was *neutral* arabinoxylan and had a β-D-1,4-linked Xyl*p* backbone. It possessed trisaccharide branches having the sequence of α-L-Ara*f*-(1 → 3)-β-D-Xyl*p*-(1 → 3)-α-L-Ara*f* (Fischer et al., 2004). Water soluble extraction from the seeds of *Plantago major* L. exhibited potent anti-complementary activity (Samuelsen, Lund, et al., 1999). Structural analysis results showed it consisted of β-D-1,4-linked Xyl*p* backbone with short side chains attached to position *O*-2 or *O*-3. The side chains consisted of β-D-Xyl*p*, α-L-Ara*f*, α-L-Ara*f*-(1 → 3)-β-D-Xyl*p* and α-D-GlcA*p*-(1 → 3)-α-L-Ara*f*. The acidic heteroxylan from *P. asiatica* also possessed anti-complementary activity, but had a different structure (Yamada et al., 1985). The highly branched and partially *O*-acetated polysaccharide consisted of β-D-1,4-linked Xyl*p* backbone, with short side chains linked to *O*-3. The short side chains were composed of β-D-Xyl*p*, α-D-GlcA*p*-(1 → 3)-α-L-Ara*f* and α-D-GalA*p*-(1 → 3)-α-L-Ara*f*.

Our previous studies showed that crude polysaccharide from the seeds of *Plantago asiatica* L. possessed antioxidant activities (Yin, Nie, Zhou, Wan, & Xie, 2010). And it had significant immuno-enhancing activity by inducing the maturation of dendritic cells (Huang, Tang, et al., 2009; Huang, Xie, et al., 2009). In the current study, polysaccharides extracted from seeds of *P. asiatica* L. were purified into three fractions (PLP-1, PLP-2 and PLP-3) by Sephacryl™ S-400 HR column chromatography. The main fraction PLP-2's structure was elucidated using oxalic acid hydrolysis, partial acid hydrolysis, methylation, GC, GC–MS, 1D and 2D NMR. 2D NMR spectroscopy included homonuclear $^{1}H/^{1}H$ correlation spectroscopy (DQF-COSY, TOCSY) and heteronulcear $^{13}C/^{1}H$ multiple-quantum correlation spectroscopy (HSQC, HMBC).

2. Experimental

2.1. Material

The seeds of *P. asiatica* L. were purchased from Ji'an County (Jiangxi Province, China) and dried before use. The species were

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identified by Dr. Cui-sheng Fan, Jiangxi University of Traditional Chinese Medical (Nanchang, China).

Dextran of different molecular weights (T-2000, T-70, T-40 and T-10 with molecular masses of 2,000,000, 70,000, 40,000 and 10,000, respectively) and Sephacryl™ S-400 HR were from Pharmacia Co. (Uppsala, Sweden). Monosaccharide standards of D-mannose (Man), L-rhamnose (Rha), D-ribose (Rib), D-galactose (Gal), D-xylose (Xyl), D-arabinose (Ara), L-fucose (Fuc) and D-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 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 reagents were of analytical grade.

2.2. Isolation and purification of polysaccharide

The extraction process was conducted according to the earlier report (Yin et al., 2010). Briefly, the seeds of *P. asiatica* L. (100 g) were defatted with ethanol (80%, v/v) and dried in the open air. They were extracted with boiling water (1000 ml) for 3 h. The residue was further extracted in the same procedure. The combined aqueous extract formed a highly viscous dispersion. It was centrifugated and filtered through a cotton cloth bag and concentrated in a rotary evaporator at 55 °C. The filtrate was added with papain (0.15%) and heated in water (60 °C) for 2 h. Then, the solution was deproteinized according Sevag method (Staub, 1965). The resulting aqueous solution was dialyzed and precipitated by ethanol at a final concentration of 80% for more than 12 h. After centrifugation, the precipitate was washed with anhydrous ethanol, dissolved in water and lyophilized to yield *P. asiatica* L. crude Polysaccharide (PLCP).

The crude polysaccharide was redissolved in distilled water and applied to a Sephacryl™ S-400 HR column (2.6 cm × 60 cm). The column was eluted with 15 mM NaCl at 1.2 ml/min and monitored by the phenol-sulfuric acid (Michel, Gilles, Hamilton, & Rebers, 1956). Fractions were concentrated, dialyzed and lyophilized according to the elution curve.

2.3. Molecular weight and monosaccharide composition analysis

The homogeneity and molecular weight was determined by HPGPC method (Yin et al., 2010), on a Waters HPLC system (UK6 injector and 515 HPLC pump, Waters, Milford, MA) equipped with a Waters Ultrahydrogel™ Linear column (7.8 mm × 300 mm), a Waters 410 differential refractometer and a Millennium^{32} workstation. Dextran standards and glucose were used to establish a standard curve.

The polysaccharide was hydrolyzed by 2 M TFA at 100 °C for 12 h and applied for monosaccharide compositions analysis using GC method (Chen, Xie, Nie, Li, & Wang, 2008). Gas chromatography was used with a DB-1701 capillary column (30 m × 0.25 mm × 0.25 μm) and a flame ionization detector (FID). An initial column temperature held at 170 °C for 2 min, then programmed at a rate of 10 °C/min to 250 °C, finally held at 250 °C for 10 min.

Protein was determined by photometric assay (Bradford, 1976) using bovine serum albumin as the standard. Uronic acid was determined by measuring the absorbance at 525 nm using the m-hydroxybiphenyl photometric procedure, with D-glucuronic acid as the standard (Blumenkrantz & Asboe-Hansen, 1973).

2.4. Methylation and GC–MS analysis

2.4.1. Reduction of polysaccharide

The reduction of the uronic acid was conducted following a procedure described by relative reports (Taylor & Conrad, 1972; Singthong, Cui, Ningsanond, & Goff, 2004), with slight modifications. PLP-2 (20 mg) was added into distilled water and reduced with 1-cyclohexyl-3-(2-morpholinoethyl)-carbodiimide methyl-p-toluenesulfonate (CMC, Sigma). The reduction procedure was repeated for more than three times until the uronic acid was reduced completely. The reduced polysaccharide (PLP-2-R) was subjected for monosaccharide compositions and methylation analysis.

2.4.2. Methylation and GC–MS analysis

Methylation analysis of polysaccharide (PLP-2 and PLP-2-R) was conducted according to the method of previous research (Ciucanu & Kerek, 1984; Nie et al., 2011) with some modifications. The dried polysaccharide was dissolved in anhydrous dimethyl sulphoxide, sonicated at 50 °C for 1.3 h, then followed by stirring at room temperature for 20 h. Methyl iodide (1 ml) was added slowly into the solution at ice bath. 0.5 h later, another 0.3 ml methyl iodide was added. The mixture was stirred for another 2.5 h. The methylated polysaccharide was first extracted with chloroform, then washed with distilled water for three times. The chloroform extract was evaporated by a stream of nitrogen.

The methylated polysaccharide was then converted into partially methylated alditol acetateds (PMAA) by hydrolysis and acetylation. The linkage analysis was conducted on an Agilent 7890–7000 A GC–MS system with a SP-2330 column (30 m × 0.25 mm, 0.2 μm film thickness, Supelco, Bellefonte, PA), equipped with an ion trap MS detector. 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. The percentage of methylated sugars was estimated as ratios of the peak areas (total ion current).

2.5. Partial hydrolysis analysis

2.5.1. Oxalic acid hydrolysis

Ara was partly removed from the polysaccharide fractions by hydrolysis with 0.05 M oxalic acid at 100 °C for 2 h followed by dialysis (Mw cut-off 3500) and lyophilizing (Cartier, Chambat, & Joseleau, 1987), named PLP-2-OA. PLP-2-OA was conducted for methylation analysis.

Table 1
Yields, sugar, protein, uronic acid contents and optical rotation of polysaccharides from the seeds of *Plantago asiatica* L.

Samples	Appearance	Yields (%)	Sugar (%)[c]	Protein (%)[c]	Uronic acid (%)[c]	Optical rotation
PLCP	Light brown, fluffy	6.2[a]	68.22 ± 1.04	1.45 ± 0.09	16.62 ± 1.33	Not determined
PLP-1	White-colour, fluffy	18.9[b]	82.08 ± 0.02	0.54 ± 0.03	17.48 ± 0.38	−21.4
PLP-2	White-colour, fluffy	52.6[b]	87.32 ± 0.13	1.16 ± 0.02	14.66 ± 0.53	−6.3
PLP-3	White-colour, fluffy	28.5[b]	79.18 ± 0.01	0.72 ± 0.04	20.13 ± 0.70	−12.3

[a] The yields was determined by comparing with raw material.
[b] The yields were compared with PLCP.
[c] Data were shown as mean, $n = 3$.

Table 2
Glycosyl-linkage compositions of PLP-2, PLP-2-OA and PLP-2-P-H.[a]

Residue linkage	Corresponding derivatives	PLP-2 (%)	PLP-2-OA (%)	PLP-2-P-H (%)
T-Linked Xyl*p*	1,5-O-Ac_2-2,3,4-Me_3-Xylitol	11.5	26.8	21.9
1,3-Linked Xyl*p*	1,3,5-O-Ac_3-2,4-Me_2-Xylitol	3.5	–	10.9
1,4-Linked Xyl*p*	1,4,5-O-Ac_3-2,3-Me_2-Xylitol	3.7	39.1	26.8
1,3,4-Linked Xyl*p*	1,3,4,5-O-Ac_4-2-Me-Xylitol	24.9	9.4	11.6
1,2,4-Linked Xyl*p*	1,2,4,5-O-Ac_4-3-Me-Xylitol	10.5	3.6	11.2
T-Linked Ara*f*	1,4-O-Ac_2-2,3,5-Me_3-Arabinitol	6.3	4.9	4.3
1,2-Linked Ara*f*	1,2,4-O-Ac_3-3,5-Me_2- Arabinitol	1.4	–	–
1,3-Linked Ara*f*	1,3,4-O-Ac_3-2,5-Me_2- Arabinitol	9.6	4.1	3.8
1,5-Linked Ara*f*	1,4,5-O-Ac_3-2,3-Me_2- Arabinitol	0.9	4.1	–
T-Linked Glc*p*	1,2,3,6-O-Ac_4-1,5-Me_2-Gluctiol	–	8.1	1.7
T-Linked GlcA*p*	1,2,3,6-O-Ac_4-1,5-Me_2-Gluctiol	14.0	–	–
1,4-Linked Glc*p*	1,4,5-O-Ac_3-2,3,6-Me_3-Glucitol	1.6	–	–
1,6-Linked Glc*p*	1,5,6-O-Ac_3-2,3,4-Me_3-Gluctiol	2.3	–	0.4
1,3,4-Linked Gal*p*	1,3,4,5-O-Ac_4-2,6-Me_2-Gluctitol	1.7	–	–
1,3,6-Linked Glc*p*	1,3,5,6-O-Ac_4-2,4-Me_2-Gluctitol	2.8	–	–
T-Linked Rha*p*	1,5-O-Ac_2-2,3,4-Me_3-Rhamnitol	1.5	–	2.5
1,3-Linked Rha*p*	1,3,5-O-Ac_3-2,4-Me_2-Rhamnitol	1.9	–	1.8
1,4-Linked Gal*p*	1,4,5-O-Ac_3-2,3,6-Me_2-Galacitol	1.4	–	1.6

–, not determined.
[a] Calculated a percentage of all partially methylated alditol acetates present, based on the peak area.

2.5.2. Partial acid hydrolysis

PLP-2 (60 mg) was hydrolyzed with 0.1 M TFA at 90 °C for 1 h. After cooling, TFA was evaporated under a stream of N_2. The hydrolysate was dissolved and dialyzed against distilled water (Mw cut-off 3500 Da, 3 × 500 ml) to obtain two fractions, higher molecular weight fraction (PLP-2-P-H) and lower molecular weight fraction (PLP-2-P-L). PLP-2-P-L and PLP-2-P-H were collected for monosaccharide compositions. PLP-2-P-H was further applied for methylation, 1D and 2D NMR analysis.

2.6. NMR spectrum

PLP-2 and PLP-2-P-H was dissolved in 99.9% D_2O. The 1D and 2D NMR spectrum of samples 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 298 K. The spectrum of ^1H NMR of PLP-2-P-H was further conducted at 323 K to obtain proton signals distributed in the area of δ 4.60–4.90 ppm.

3. Results and discussion

3.1. Isolation and purification of polysaccharide

The PLCP was obtained from the seeds of *P. asiatica* L. by hot water extraction in a yield of 6.2%. The PLCP was separated through a Sephacryl™ S-400 HR column (2.4 cm × 60 cm) into three fractions (PLP-1, PLP-2 and PLP-3), which was detected by the phenol-sulfuric acid assay. As shown in Table 1, the main fraction of PLCP was PLP-2 with a high yield of 52.6% (18.9% for PLP-1, 28.5% for PLP-3). Among the three fractions, PLP-2 had the highest sugar contents (87.32%). It was 82.08% and 79.18% for PLP-1 and PLP-3, respectively. The sugar contents of three fractions purified by gel permeation were higher than that of PLCP, indicating further purification for the crude polysaccharide was necessary. Protein determination results showed that all of them were in low contents (only 0.54% of PLP-1, 1.16% of PLP-2 and 0.72% of PLP-3). The uronic acid contents of PLP-2 were the lowest (14.66%). It was 17.48% for PLP-1 and 20.13% for PLP-3. The optical rotation values of the three fractions were −21.4, −6.7 and −12.3, respectively. The negative value indicated that primary pattern of polymers were β-linked configuration. The following analysis was focused on the main fraction PLP-2.

PLP-2 was eluted as a single peak from HPLC with Ultrahydrogel™ linear column (not shown). The weight average molecular weight was about 1849 kDa based on a column calibration. Samuelsen, Paulsen, Wold, Knutsen, and Yamada (1998) reported the molecular weight of polysaccharide from the leaves of *Plantago major* L. was 77,000–80,000. And Al-Assaf isolated a polysaccharide from Ispaghula husk which had molecular weight ranging from 10–20 × 10^6 (Al-Assaf et al., 2003). These discrepancies cannot be easily resolved as the samples reported in these studies were from different sources and often the methods used are not consistent. The different treatment in extracting and purifying process may also lead to fractions with different molecular weight. The molecular weight of PLP-2 determined in the current study was similar to the result by Edwards and co-workers who reported 2.2 × 10^6 Da for the water extracted polysaccharide (Edwards, Chaplin, Blackwood, & Dettmar, 2003).

3.2. Monosaccharide composition

The monosaccharide composition of PLP-2 was analyzed by GC. Compared with the monosaccharide standards, PLP-2 was mainly composed of Ara (32.2%) and Xyl (61.1%). There were trace amounts of Rha, Man, Glc and Gal. Rha, Ara, Xyl, Man, Glc and Gal were found to be present in a molar ratio of 0.05:1.00:1.90:0.05:0.06:0.10. In other words, PLP-2 was mainly arabinoxylan. It was similar to previous reports on polysaccharides from the *Plantago* family (Edwards et al., 2003; Guo, Cui, Wang, & Christopher Young, 2008; Samuelsen, Cohen, Paulsen, Brull, & Thomas-Oates, 1999). After reduction with CMC–$NaBH_4$ completely, the carboxyl-reduced derivative PLP-2-R was obtained. Composition analysis indicated the presence of Rha, Ara, Xyl, Man, Glc and Gal was in a molar ratio of 0.09:1.00:1.97:0.01:0.45:0.08. Compared with PLP-2, the ratio of Glc increased from 0.06 to 0.45 which indicated GlcA was the uronic acid in PLP-2.

3.3. Methylation and GC–MS

Methylation analysis gives information on the positions at which sugar residues are substituted and their relative abundance in the polysaccharide. The polysaccharide was methylated completely, which was confirmed by the IR spectrum.

The methylation analysis results are presented in Table 2. PLP-2 was highly branched. It was consisted of 24.9% of 1,3,4-linked

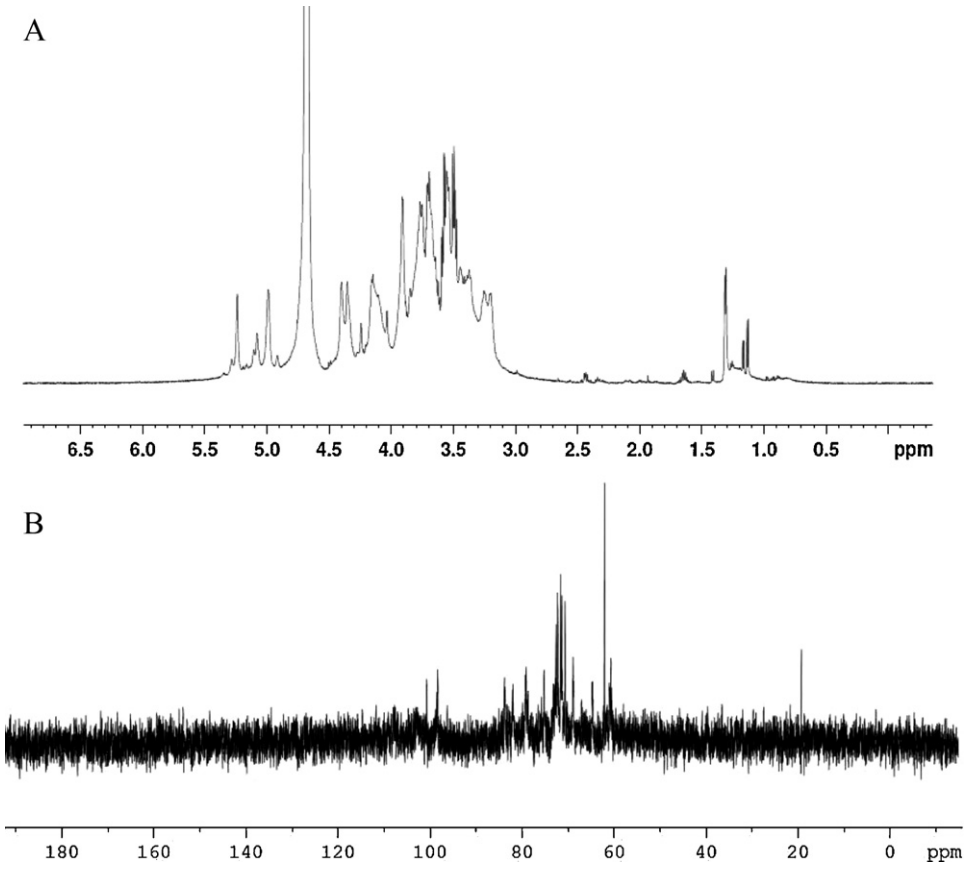

Fig. 1. ^{1}H (600.1 MHz) and ^{13}C NMR (151.0 MHz) spectrum of PLP-2.

Xyl*p*, 10.5% of 1,2,4-linked Xyl*p*, 1.7% of 1,3,4-linked Gal*p* and 2.8% of 1,3,6-linked Glc*p*. The main branch units were 1,2,4-linked Xyl*p* and 1,3,4-linked Xyl*p*, branching to *O*-2 and *O*-3 positions respectively. Several studies reported that there was 1,2,3,4-linked Xyl*p* residue, however, it was considered as a single branch point due to the possibility that the lone free hydroxyl group on these residues escaped in the methylation process (Fischer et al., 2004). There was no 1,2,3,4-linked Xyl*p* detected in the study. That indicated the methylation process taken in this study was effective. According to literatures (Fischer et al., 2004; Guo et al., 2008; Samuelsen, Cohen, et al., 1999), the linkage of 1,3-linked Ara*f* could be attributed to the side chain. As shown in Tab.2, PLP-2 contained non-reducing ends Ara (6.3%) and Xyl (11.5%). Resides of T-linked Ara*f* and T-linked Xyl*p* indicated that terminal xylo-pyranosyl and arabino-furanosyl existed in PLP-2. Uronic acid in PLP-2 was presented as non-reducing end-units of GlcA*p* (14.0%).

The results suggested that Xyl was present only in pyranosyl ring form and Ara was in the furanosyl form which was similar to other polysaccharide from other *Plantago* family (Fischer et al., 2004; Guo et al., 2008).

3.4. Partial hydrolysis

The above methylation analysis results showed that the structure of PLP-2 was complex. It is necessary to utilize other methods, such as partial hydrolysis analysis, to elucidate its structural information.

3.4.1. Oxalic acid hydrolysis

Ara residues can be removed selectively from the polysaccharide by oxalic acid, and the position of Ara in the original polysaccharide can be determined by methylation of the remaining polysaccharide (Cartier et al., 1987; Samuelsen, Lund, et al., 1999). The methylation analysis results are showed in Table 2.

After oxalic acid hydrolysis, a significant increase of 1,4-linked Xyl*p* and a decrease of 1,2,4-linked Xyl*p* and 1,3,4-linked Xyl*p* were observed. This confirmed that some Ara was linked to *O*-3 or *O*-2 of 1,4-linked Xyl*p*. There were no 1,3-linked Xyl residues in PLP-2-OA, while T-linked Xyl*p* increased. This indicated that Ara was linked to position *O*-3 of some terminal Xyl units which were most likely located in side chains. The relatively high proportion of non-reducing ends of Xyl before (11.5%) and after (26.8%) hydrolysis indicated that PLP-2 either was of a relatively high amounts of branching points and/or large Xyl side chains was present.

3.4.2. Partial acid hydrolysis

Partial degradation of polysaccharide by acid hydrolysis is based on the fact that some glycosidic linkages are more labile to acid than others. To study the linkages between backbone and side chains of polysaccharide, PLP-2 was hydrolyzed with 0.1 M TFA to produce two sub-fractions: lower molecular weight fraction (PLP-2-P-L) and higher molecular weight fraction (PLP-2-P-H).

The monosaccharide compositions indicated that the molar ratio of Rha, Ara, Xyl, Man, Glc and Gal in PLP-2-P-H was 0.15:1.00:7.73:0.12:0.14:0.53. PLP-2-P-L consisted of Ara, Xyl, Glc and Gal, in a molar ratio of 1.00:1.84:0.01:0.04. The amount of Ara in PLP-2-P-H decreased considerably compared with PLP-2, whereas the amount of Xyl increased. That suggested Ara was probably in the branch chains and Xyl was in the back bone. For the lower molecular weight fraction, PLP-2-P-L, the sugar compositions showed that Ara and Xyl were the main constituents. These results confirmed

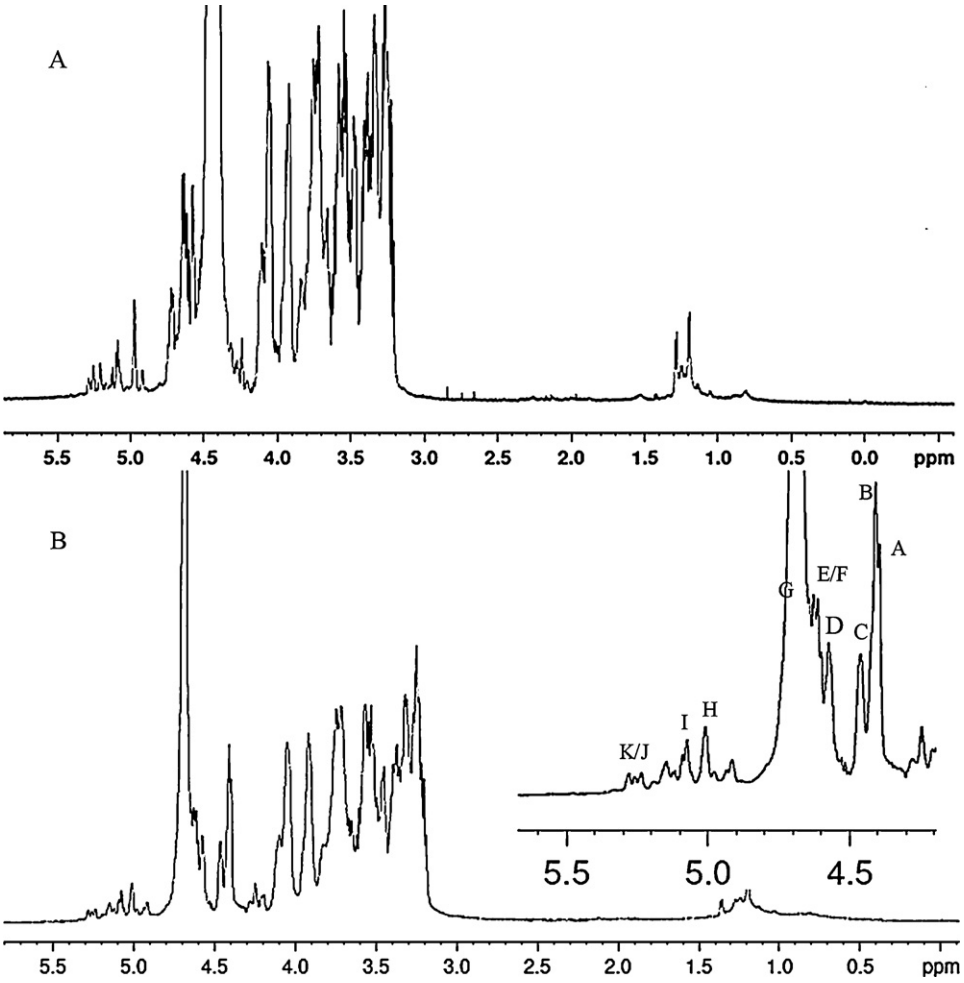

Fig. 2. ^{1}H NMR (600.1 MHz) spectrum of PLP-2-P-H determined at 323 K (A) and 298 K (B).

the observation by oxalic acid hydrolysis which showed part of Ara and Xyl were in the branch.

Methylation analysis results of PLP-2-P-H are shown in Table 2. 1,4-linked Xyl*p* was the majority residue in PLP-2-P-H, which indicated that it probably was in the backbone of the chain. The most branched sugar residues were 1,2,4-linked Xyl*p* and 1,3,4-linked Xyl*p* which accounted for 11.6% and 11.2% respectively. 1,3,4-linked Xyl*p* decreased from 23.7% to 11.6%, suggesting 1,4-linked Xyl*p* were mainly derived from 1,3,4-linked Xyl*p*. The majority of the terminal units were T-linked Xyl*p* (21.9%) and T-linked Ara*f* (4.3%), with small amount of T-linked Rha*p* (2.5%) and T-linked Glc*p* (1.7%). Other sugar residues included 1,3-linked Xyl*p* (10.9%), 1,3-linked Ara*f* (3.8%), 1,3-linked Rha*p* (1.8%), 1,4-linked Gal*p* (1.6%), 1,6-linked Glc*p* (0.4%) and trace 1,6-linked Gal*p*.

Table 3
Chemical shifts assignment of ^{1}H and ^{13}C NMR spectrum of PLP-2-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	4.39	3.21	3.38	3.58	3.91	3.23
β-1,4-linked Xyl*p*	101.29	72.20	75.03	82.78	64.64	
B	4.41	3.24	3.50	3.71	3.91	3.25
β-1,4-linked Xyl*p*	101.29	72.20	73.11	75.87	64.64	
C	4.46	3.45	3.78	3.52	3.84	3.32
β-1,2,4-linked Xyl*p*	101.07, 101.09	79.83	72.61	78.70	62.19	
D	4.57	3.25	3.40	3.57	3.90	3.28
β-T-linked Xyl*p*	99.57	73.11	74.89	70.44	64.22	
	4.60/103.17	–	–	–	–	–
E	4.61	3.27	3.63	3.93	3.96	3.28
β-1,3,4-linked Xyl*p*	102.97	72.50	82.78	75.87	64.22	
F	4.63, 4.64	3.32	3.52	3.73	4.05	3.31
β-1,3,4-linked Xyl*p*	102.86	72.81	78.70	75.76	62.38	
G	4.71	3.31	3.53	3.65	3.95	3.28
β-1,3-linked Xyl*p*	102.66	73.40	78.70	67.08	64.22	

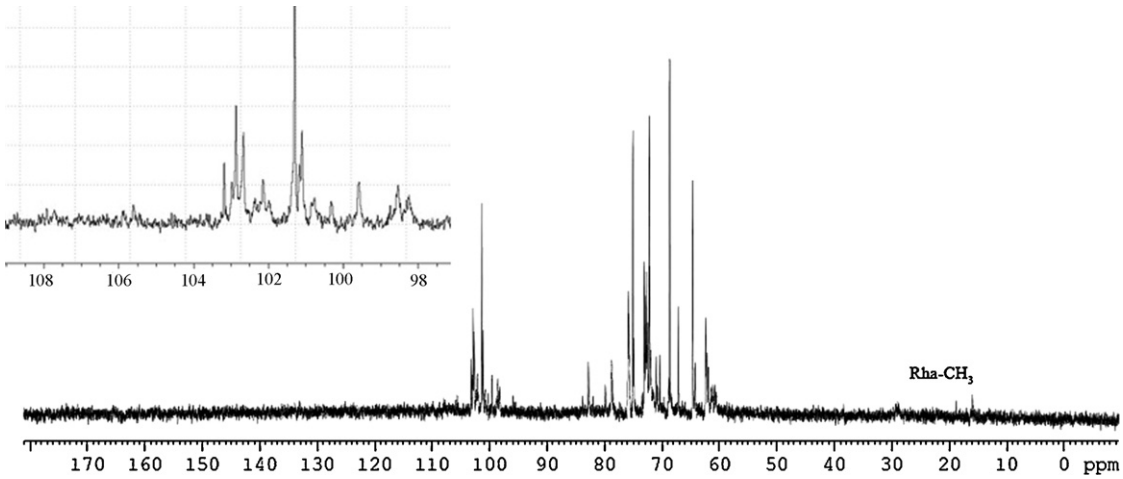

Fig. 3. ^{13}C NMR (151.0 MHz) spectrum of PLP-2-P-H.

3.5. NMR spectroscopy

It is challenging to get a high resolution NMR spectrum for viscosity polysaccharide, especially for the gel like polysaccharide from *Plantago* family, even in a higher temperature (Fischer et al., 2004; Samuelsen, Lund, et al., 1999). As seen in Fig. 1, there is some useful information from ^{1}H NMR of PLP-2. But the signal/noise ratio of ^{13}C NMR was too low to provide meaningful results. Therefore, the partial acid fraction PLP-2-P-H was then conducted for NMR analysis.

3.5.1. NMR analysis of PLP-2

Monosaccharide compositions results of PLP-2 may allow us to assume PLP-2 was a kind of arabinoxylans. Fig. 1 shows the ^{1}H NMR spectrum of PLP-2. Compared with present studies, the chemical shifts of anomer hydrogen of arabinoxylan appeared between δ 4.4–5.5 ppm (Gruppen et al., 1992). Usually, 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; Ebringerová, Hromádková, & Berth, 1994; Hoffmann, Kamerling, & Vliegenthart, 1992). In this case, it could explain why the optical rotation of PLP-2 was only −6.7. There was no absorption between δ 6–8 ppm in Fig. 3, implying no phenolic proportion and ferulic acid in PLP-2 (Colquhoun, Ralet, Thibault, Faulds, & Williamson, 1994). The signals of δ 1.31 and 1.30 ppm were assigned to Rha*p* residues, indicating there might be two kinds of linkage patterns. Signals for C-1 α or C-1 β from the reducing end-groups at δ 92.8 or 97.3 ppm were not detected in the spectra, indicating a high degree of branching (Brillouet & Joseleau, 1987), which confirmed the results from methylation analysis of high amounts of 1,2,4-linked and 1,3,4-linked Xyl*p* residues in the polysaccharide. In the high field, there was some absorption from Rha*p* residues at δ 19.17 ppm.

3.5.2. NMR analysis of PLP-2-P-H

The ^{1}H and ^{13}C NMR spectrum of PLP-2-P-H are shown in Figs. 2 and 3. According to the characteristic signals, the ^{1}H and ^{13}C spectrum of PLP-2-P-H was assigned using HSQC (Fig. 4) and HMBC, DQF-COSY and TOCSY as showed in Table 3. Peaks in ^{1}H NMR anomeric region were designed A, B, C, ..., K in decreasing chemical shifts order. All the ^{1}H and ^{13}C signals of A, B, C, D, E, F and G were assigned completely. Others were partially assigned. The long-range HMBC data is summarized in Table 4.

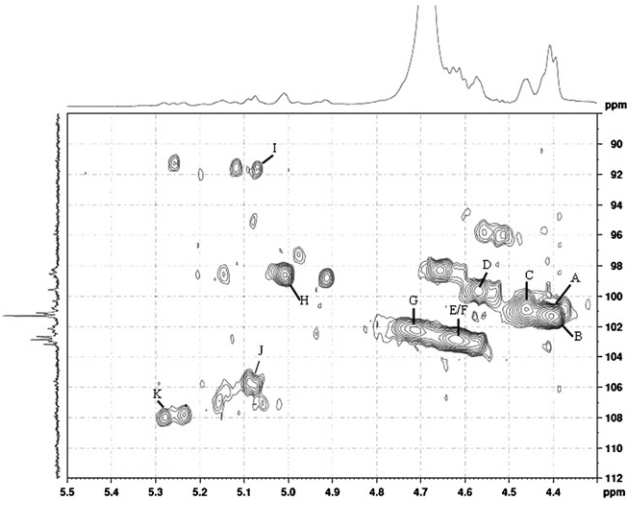

Fig. 4. Part of HSQC spectrum of PLP-2-P-H (anomeric region).

3.5.2.1. β-1,4-linked Xylp (A and B). According to the methylation analysis, 1,4-linked Xyl*p* was the most abundant residue. In the ^{1}H NMR spectrum of PLP-2-P-H, the peak at δ 4.39 and 4.41 ppm were the most intense peaks in the anomeric region, both for their anomeric carbon at δ 101.29 ppm from HSQC. By comparing with literature data and peak intensity, this peak was tentatively assigned to the anomeric proton of β-1,4-linked Xyl*p* (Bock, Pedersen, & Pedersen, 1984; Hoffmann et al., 1992; Du et al., 2009). The overlap peaks at δ 4.39 and 4.41 ppm indicated residue 1,4-linked Xyl*p* probably presented in two different chemical environment. Cross-peaks between H-1 and H-2, and between H-2 and H-3, were observed in the DQF-COSY spectrum. The H-4 and H-5 resonances were assigned from the TOCSY spectrum. The ^{13}C chemical shifts of residue A were obtained from HSQC, and they were all in consistent with the previous information (Bock et al., 1984; Hoffmann et al., 1992; Du et al., 2009).

3.5.2.2. β-1,2,4-linked Xylp (C). Residue C had anomeric chemical shift at δ 4.46 ppm, indicating it was a β-linked residue. The assignment of H-2 was conducted by its correlation with H-1 signal at δ 4.46 ppm in the DQF-COSY spectrum. In the same way, H-3 was assigned from its correlation with H-2. However, the assignment of H-4 and H-5 from QDF-COSY spectrum became difficult due to multiple signals. The specific allocation of H-4

and H-5 resonances of residue C was supported by TOCSY spectrum. The corresponding ^{13}C NMR chemical shifts were obtained form the cross peaks of H-1–C-1, H-2–C-2...H-5–C-5 in the HSQC spectrum. By comparing with previous reports (Bendahou, Dufresne, Kaddami, & Habibi, 2007; Fischer et al., 2004), especially ^{13}C NMR data, residue B was assigned to be β-1,2,4-linked Xyl*p*.

3.5.2.3. β-T-linked Xylp (D). Residue D had anomeric chemical shifts at δ 4.57 ppm. The chemical shifts from H-1 to H-5 were assigned from DQF-COSY, and confirmed by TOCSY spectrum. The chemical shift from C-1 was assigned from HSQC. By comparing literature data, residue D was tentatively assigned to the β-T-linked Xyl*p*. The specific allocation of C-2 and C-5 resonances of residue D was assigned from HSQC. The C-3 and C-4 chemical shifts were supported by literatures (Bock et al., 1984; Fischer et al., 2004; Hoije et al., 2006). The anomeric chemical shifts at δ 4.60 and 103.17 ppm (from HSQC) were also from β-T-linked Xyl*p*. But it was not further analyzed because of the multiple overlapping signals in DQF-COSY and TOCSY.

3.5.2.4. β-1,3,4-linked Xylp (E and F). The proton chemical shifts at the region δ 4.63 and 4.64 ppm were assigned to residue F, since both of their carbon anomeric chemical shifts were δ 102.86 ppm from HSQC. The proton chemical shift at δ 4.61 ppm was assigned to residue E and its carbon anomeric chemical shift was δ 102.97 ppm. Their chemicals shifts of H-2 to H-5 were obtained from DQF-COSY, supported with TOCSY. The chemicals shifts of C-2 to C-5 were confirmed from HSQC spectra. The downfield shifts of the C-3 (δ 82.78 ppm for E, δ 78.70 ppm for F) and C-4 (δ 75.78 ppm for E, δ 75.76 ppm for F) suggested their *O*-3 and *O*-4 were substituted. That suggested they were in β-1,3,4-linked styles. The methylation analysis results showed only Xyl was 1,3,4-linked. The chemical shifts for H and C were in agreement with previous reports of 1,3,4-linked Xyl*p* (Apirattananusorn et al., 2008; Pastell, Virkki, Harju, Tuomainen, & Tenkanen, 2009). It indicated that residues of E and F were β-1,3,4-linked Xyl*p*. The different chemical shifts of proton and carbon were probably because of their different chemical environment.

3.5.2.5. β-1,3-linked Xylp (G). Residue G had an anomeric chemical shift at δ 4.71 ppm. Cross-peaks at δ 4.71/3.31 ppm and δ 3.31/3.53 ppm were detected in the DQF-COSY spectrum, since δ 4.71 ppm corresponded to H-1, δ 3.31 and 3.53 ppm signals were assigned to H-2 and H-3, respectively. The ^1H resonances for H-4, H-5 and H-5′ were assigned from the TOCSY and DQF-COSY spectrum. The carbon signals from C-1 to C-5 of residue G were identified from HSQC. From published data (Fischer et al., 2004; Rantanen et al., 2007; Samuelsen, Lund, et al., 1999), the chemical shifts of G (both proton and carbon signals) indicated it was a β-1,3-linked Xyl*p*.

3.5.2.6. Identification of residues H, I, J and K. Residues H, I, J and K had anomeric chemical shifts at δ 5.01, 5.07, 5.09 and 5.26 ppm, respectively. Because of weak signals from the spectrum, they could only be partially assigned. Residues J and K were assigned to signals of anomeric proton from α-T-Ara*f* and α-1,3-linked Ara*f* respectively, based on literature values (Kang et al., 2010). Residue I had an anomeric chemical shift at δ 5.07 ppm. Its H-2 chemical shift was 4.24 ppm from DQF-COSY. Its carbon anomeric chemical shift was δ 95.94 ppm. Therefore, residue I was assigned to α-1,3-linked Rha*p*. There was a large amount of T-GlcA*p* in PLP-2 (14.0%). We could also assume that there was still some T-GlcA*p* in PLP-2-P-H, which was confirmed by FT-IR spectra of absorption at 1720.1 and 1637.7 cm^{-1} (Gnanasambandam & Proctor, 2000). Compared with ^1H NMR spectrum of PLP-2, the signal δ 5.01 ppm of PLP-2-P-H decreased greatly. This observation suggested residue H was α-T-GlcA*p*. Its H-2 chemical shift was δ 3.54 ppm from DQF-COSY.

As a result of all the structural information descried, a possible structure for PLP-2 is proposed as follows:

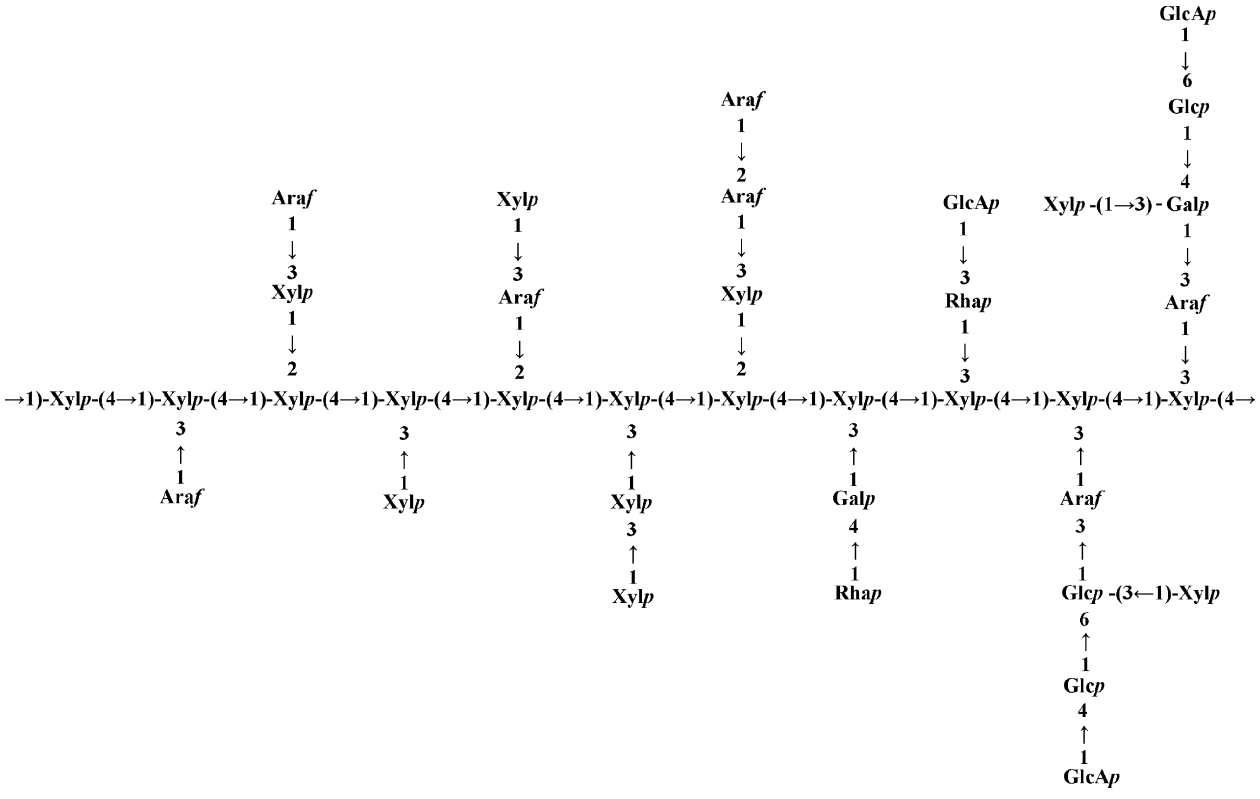

Table 4
The significant connectivities observed in HMBC spectrum for anomeric proton/carbon of the sugar residues of PLP-2-P-H.

Sugar residues	Sugar linkage	H-1/C-1	Observed connectivities		
		δ_H/δ_C	δ_H/δ_C	Residue	Atom
A/B	1,4-β-linked Xyl*p*	4.39/4.41	75.87	B	C-4
			75.87	E	C-4
		101.29	3.93	E	H-4
C	1,2,4-β-linked Xyl*p*	4.46	75.76	F	C-4
			75.87	B	C-4
			75.87	E	C-4
		101.09/101.17	3.93	E	H-4
D	T-β-linked Xyl*p*	4.57	79.83	C	C-2
		99.57	3.45	C	H-2
		4.60	82.78	E	C-3
		103.17			
E/F	1,3,4-β-linked Xyl*p*	4.61/4.63/4.64	82.78	A	C-4
		102.97/102.86	3.93	E	H-4
G	1,3-β-linked Xyl*p*	4.71/4.72	78.70	C/E	C-4
		102.66	3.45	B	H-2

In summary, PLP-2 is consisted of a β-1,4-linked Xyl*p* backbone with side chains attached to *O*-2 or *O*-3, which was similar to other studies (Fischer et al., 2004; Samuelsen, Lund, et al., 1999; Yamada et al., 1985). These results suggests that polysaccharide from different *Plantago* species have similar structure. However, there were some differences in side chains. Fischer et al. (2004) reported arabinoxylan from *Plantago ovata* Forsk as neutral polysaccharides, while Samuelsen, Lund, et al. (1999) showed a polysaccharide had α-GlcA in side areas. Yamada et al. (1985) reported there were α-GalA and α-GlcA. In our study, only α-GlcA was found as T-linked in side chains. PLP-2 showed a more complex structure in side chains. 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*. The structural difference between PLP-2 and other polysaccharide from other *Plantago* family was probably because of species and habitat difference.

4. Conclusion

In the current study polysaccharide was extracted from the seeds of *P. asiatica* L. with hot water and separated into three fractions PLP-1 (18.9%), PLP-2 (52.6%) and PLP-3 (28.5%) by Sephacryl™ S-400. The main fraction PLP-2's structure was elucidated by the combination of oxalic acid hydrolysis, partial acid hydrolysis, methylation, combined with GC, GC–MS, 1D and 2D NMR. PLP-2 was composed of Gal, Rha, Ara, Xyl, Man, Glc and Gal in a molar ratio of 0.05:1.00:1.90:0.05:0.06:0.10. Its uronic acid was GlcA. PLP-2 was highly branching heteroxylan which consisted of a β-1,4-linked Xyl*p* backbone with side chains attached to *O*-2 or *O*-3 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*, etc. This paper provides detail structure information of polysaccharide from the seeds of *P. asiatica* L.

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