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Structure characterization of high molecular weight heteropolysaccharide isolated from *Artemisia sphaerocephala* Krasch seed

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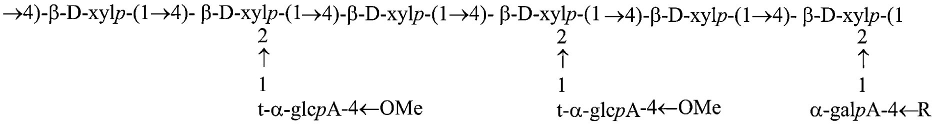
ABSTRACT

The structure of a high molecular weight heteropolysaccharide (60P) isolated from *Artemisia sphaerocephala* Krasch was elucidated using methylation analysis and 2D NMR spectroscopy. 60P (Mw: 551 kDa) was isolated from *A. sphaerocephala* Krasch (ASK) seeds, using hot water extraction and ammonium sulphate salt precipitation. Methylation and GC–MS analysis indicated that 60P was mainly composed of three types of sugar residues: 4-Xyl*p* (27.8%), 2,4-Xyl*p* (26.0%) and T-Glc*p*A (22.5%); small amount of 4-Gal*p*A, 4-Glc*p*, T-Ara*f*, T-Ara*p* and 4, 6-Gal*p*A were also detected with comparable molar ratio. Based on the results of monosaccharide composition, methylation analysis and NMR spectroscopy, a proposed structure is shown below:



$$\begin{array}{llllll}
\rightarrow 4)\text{-}\beta\text{-D-xyl}p\text{-}(1\rightarrow 4)\text{-} & \beta\text{-D-xyl}p & \text{-}(1\rightarrow 4)\text{-}\beta\text{-D-xyl}p\text{-}(1\rightarrow 4)\text{-} & \beta\text{-D-xyl}p & \text{-}(1\rightarrow 4)\text{-}\beta\text{-D-xyl}p\text{-}(1\rightarrow 4)\text{-} & \beta\text{-D-xyl}p\text{-}(1 \\
 & 2 & & 2 & & 2 \\
 & \uparrow & & \uparrow & & \uparrow \\
 & 1 & & 1 & & 1 \\
 & \text{t-}\alpha\text{-glc}p\text{A-4}\leftarrow\text{OMe} & & \text{t-}\alpha\text{-glc}p\text{A-4}\leftarrow\text{OMe} & & \alpha\text{-gal}p\text{A-4}\leftarrow\text{R}
\end{array}$$

R represents one of the following groups: T-Ara*f*, T-Ara*p*, T-Ara*f* → 3-Ara*f* (1→ and T-Ara*p* → 3-Ara*f* (1→.

1. Introduction

Recently, there is a increased interest in *A. sphaerocephala* Krasch polysaccharide (ASKP) as a result of its beneficial functional and biological properties. Previous animal experiments indicated that ASKP gum was able to alleviate hyperglycemia, hyperlipemia and insulin resistance of streptozotocin-induced type 2 diabetic rats and could result in a significant decrease in blood glucose levels in the diabetic rats (Hu et al., 2011). In addition, its potent antioxidant effect was demonstrated on the diabetic rats (Xing, Zhang, Hu, Wu, & Xu, 2009; Zhang et al., 2007).

In our previous study, ASKP was isolated and fractionated into two fractions, 60P (M_W: 551 kDa) and 60S (M_W: 39 kDa) (Guo et al., 2010). Chemical analysis showed that 60P was composed of 55.4% neutral sugar and 25.8% acid sugar, while in 60S, the percentage of neutral sugar and acidic sugar were 87.1% and 10.4%, respectively. Monosaccharide composition test indicated that 60S was composed of glucose (38.3%), mannose (28.1%), galactose (24.2%) and arabinose (9.4%); while the 60P fraction contained mainly xylose (80.5%), arabinose (10.9%), glucose (5.0%) and galactose (2.3%), with a small amount of rhamnose (1.2%) (Guo et al., 2010).

The objective of current study was to elucidate the fine structure of 60P using methylation analysis and 2D NMR spectroscopy including homonuclear ^{1}H/^{1}H correlations spectroscopy (COSY, TOCSY), heteronuclear ^{13}C/^{1}H multiple-quantum coherence spectroscopy (HMQC) and heteronuclear multiple bond correlation spectroscopy (HMBC).

2. Experimental

2.1. Isolation and fractionation of 60P

60P fraction was isolated from *A. sphaerocephala* Krasch seeds using water extraction (1:400 w/v, 70 °C) for 24 h, followed by 60% (w/v) ammonium sulphate precipitation at room temperature. The precipitate was collected and designated 60P in the present study (Guo et al., 2010).

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Table 1
Methylation analysis data of 60P.

RT	Sugar derivative	Linkage pattern	Mol ratio[a]
9.395	2,3,5-Me$_3$-Ara	T-Ara*f*	1.7%
11.461	2,3,4-Me$_3$-Ara	T-Ara*p*	1.1%
14.635	2,3,4,6-Me$_4$-GlcA	T-Glc*p*A	22.5%
15.238	2,4,5-Me$_3$-Ara	3-Ara*f*	NO[b]
18.346	2,3-Me$_2$-Xyl	4-Xyl*p*	27.8%
20.073	Rha-(OAc)$_5$	2,3,4-Rha*p*	4.2%
21.325	2,3,6-Me$_3$-Man	4-Man*p*	1.2%
22.432	2,3,6-Me$_3$-GalA	4-Gal*p*A	5.6%
22.935	2,3,6-Me$_3$-Glc	4-Glc*p*	2.8%
24.123	3-Me-Xyl	2,4-Xyl*p*	26.0%
25.76	2,3-Me$_2$-GalA	4,6-Gal*p*A	1.3%

[a] Relative molar ratio, calculated from the ratio of peak areas.
[b] Not obtained due to overlap with the peak of T-Glc*p*A.

2.2. Methylation analysis

The high percentage of uronic acids in 60P fraction created difficulties for methylation analysis. In the current study, uronic acid was first reduced into neutral sugars prior to methylation analysis. The related steps are described as follows.

2.2.1. Reduction of uronic acids

The reduction of the uronic acids was conducted following the procedure described earlier (Taylor & Conrad, 1972; York, Darvill, McNeil, Stevenson, & Albersheim, 1986) with a few modifications. The acidic polysaccharide (5 mg) was dissolved in deuterium oxide D$_2$O (2 mL). To the solution, 50 mg of 1-cyclohexyl-3-(2-morpholinoethyl)-carboiimidemethyl-p-toluenesulfonate was added while using 0.1 mol/L HCl in D$_2$O to keep the pH at 4.75. The solution was left for 1 h under stirring, followed by adding 5 mL of sodium borodeuteride (160 mg/mL) dropwise over a period of 1 h, and the reaction mixture pH was maintained at 7.0, using 2.0 mol/L HCl in D$_2$O during the reduction reaction. The reaction was allowed to continue with constant stirring for 0.5 h at pH 7.0 after the addition of sodium borodeuteride, then the solution pH was brought back to 4.0. The reduced polysaccharide was separated from salts by dialysis against distilled water overnight at 25 °C (3500 Da molecular weight cut off), then lyophilized. The polysaccharide was re-dissolved in 1 mL distilled water and 0.5 mL 10% acetic acid in methanol was added. The mixture was dried under a stream of nitrogen to remove boric acid. Another 1 mL of 10% acetic acid in methanol was added to the residue and evaporated using nitrogen. This process was repeated 3–4 times to ensure that most of the boric acid was removed. Finally, a few drops of methanol were added and the solution was evaporated (two times) to remove any boric acid remaining.

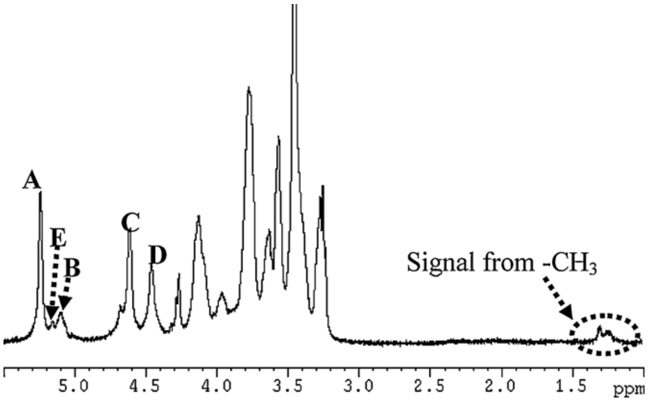


Fig. 1. A part of the ^{1}H NMR spectrum of polysaccharide 60P isolated from ASK (60 °C in D$_2$O).

2.2.2. Methylation analysis

Methylation analysis of 60P was conducted using the method of Ciucanu and Kerek (1984). The samples after reduction were dissolved in DMSO and sonicated at 40 °C for 2 h and transferred to a 70 °C water bath for 2 h with constant stirring, followed by stirring at room temperature for an additional 12 h to ensure a complete dissolution. After that, dry sodium hydroxide powder was added to the solution under constant stirring at room temperature for 3 h, followed by 2.5 h constant stirring after adding 0.3 mL methyl iodide. The mixture was extracted with 1 mL methylene chloride, passed through a sodium sulphate column, and then dried with nitrogen gas. The methylated polysaccharide was hydrolyzed by adding 0.5 mL 4 M trifluoroacetic acid (TFA) to the sample in a test tube and sealing the tube, heating at 100 °C for 6 h, cooling and then drying with N$_2$. The sample was then dissolved with distilled water (0.3 mL), and the hydrolysate was reduced using 5 mg sodium borodeuteride and acetylated with acetic anhydride (0.5 mL) for 2 h. Aliquots of the resultant partially methylated alditol acetates (PMAA) were injected into a GC–MS system (ThermoQuest Finnigan, San Diego, CA) fitted with a SP-2330 (Supelco, Bellefonte, PA) column (30 mL × 0.25 mm, 0.2 mm film thickness, 160–210 °C at 2 °C/min, then 210–240 °C at 5 °C/min) and an ion trap MS detector.

2.3. NMR studies

80 mg 60PE sample (60P with protein removed) (Guo et al., 2010) was dissolved in 4 mL D$_2$O at 70 °C with stirring for 2 h, and then freeze dried. This procedure was repeated three times. Samples were then dissolved in 3 mL D$_2$O and ready for testing. High-resolution ^{1}H and ^{13}C NMR spectra were recorded at 500.13 and 125.78 MHz, respectively, on a Bruker ARX500 NMR spectrometer operating at 60 °C. A 5 mm inverse geometry ^{1}H/^{13}C/^{15}N probe was used. Chemical shifts are reported relative to trimethylsilyl

Table 2
^{1}H NMR and ^{13}C NMR chemical shifts of the polysaccharide (60P) isolated from ASK recorded in D$_2$O at 60 °C.

Sugar residue	H1/C1	H2/C2	H3/C3	H4/C4	H5/C5	H5′	H6/C6	-OMe
T-α-D-glc*p*A	5.22	3.58	3.75	3.26	4.26			3.46
A	98.0	72.1	73.0	83.0	73.0		177	60.8
4-α-D-gal*p*A	5.08	3.77	3.97	4.42	4.65			
B	98.0	69	69.5	78.3	72.2		177	
2,4-β-D-xyl*p*	4.61	3.42	3.63	3.79	4.1	3.44		
C	101.8	77.2	73.5	77.1	63.2			
4-β-D-xyl*p*	4.44	3.28	3.57	3.78	4.14	3.41		
D	102.5	73.1	74.1	76.7	62.5			
T-β-L-Ara*f*	5.17	4.06[a]	4.11[a]	3.82[a]	3.68[a]	–[b]		
E	–	–	–	–	–	–		

[a] Found in TOCSY, interchangeable.
[b] Not detected.

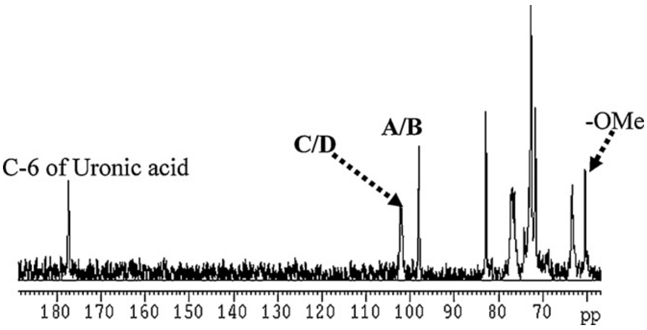


Fig. 2. A part of the ^{13}C NMR spectrum of polysaccharide 60P isolated from ASK (60 °C in D_2O).

propionate (TSP) in D_2O for ^{1}H (0.0 ppm, external standard) and 1,4-dioxane in D_2O for ^{13}C (66.5 ppm, external standard). Homonuclear ^{1}H/^{1}H correlation spectroscopy (COSY, TOCSY) and heteronuclear ^{1}H/^{13}C correlation experiments (HMQC, HMBC) were run using the standard Bruker pulse sequence.

3. Results and discussion

The results of methylation analysis of the 60P are presented in Table 1; three main alditol acetates peaks were observed in GC profile which were assigned as 2,3-Me_2-Xyl (27.8%), 3-Me-Xyl (26.0%) and 2,3,4,6-Me_4-GlcA (22.5%) indicating the presence of 4-xyl*p*, 2,4-xyl*p*, and T-Glc*p*A in 60P fraction. Sugar residues of 4-Gal*p*A, 4-Glc*p*, T-Ara*f*, T-Ara*p* and 4, 6-Gal*p*A were also detected with comparable amount (Table 1). The methylation analysis results indicated that the main sugar residues were xylose and glucuronic acid, which was consistent with the composition analysis described by Guo et al. (2010). The total percentage of terminal sugar (T-Glc*p*A, T-Ara*f* and T-Ara*p*) is 25.3%, which matched well with the branched portion (2,4- Xyl*p*: 26.0%). The degree of branching was 51.3% based on the calculation method of Hawker, Lee, and Frechet (1991). Sugar residues with molar ratio less than 1% were not included in Table 1.

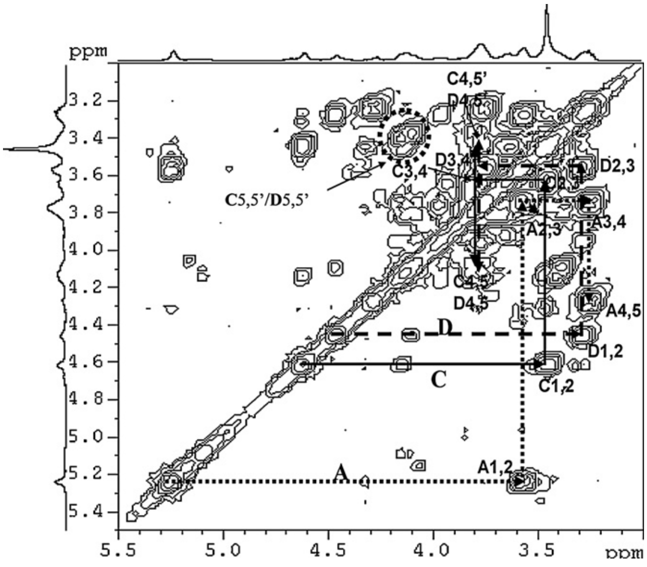


Fig. 3. A part of the COSY spectrum of polysaccharide 60P isolated from ASK (60 °C in D_2O). Proton correlations of sugar residues **A**, **C** and **D** were labelled.

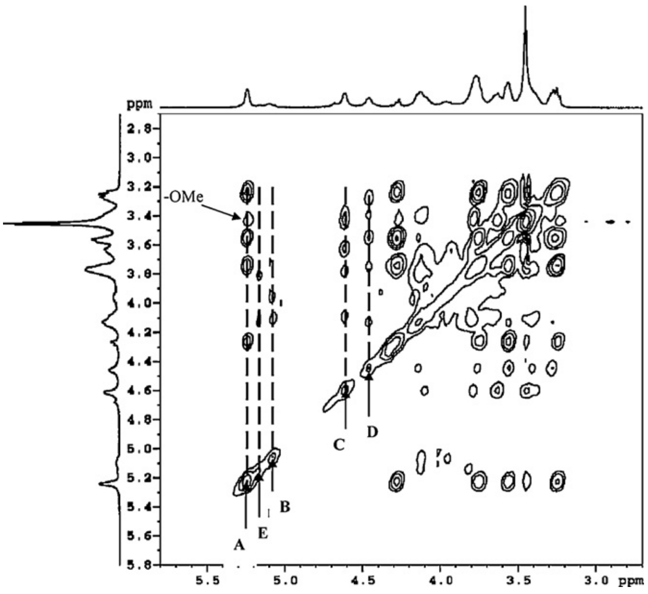


Fig. 4. A part of the TOCSY spectrum of polysaccharide 60P isolated from ASK (60 °C in D_2O).

The 500 MHz ^{1}H NMR spectrum of 60P fraction (Fig. 1) showed five anomeric proton signals at 5.22, 5.08, 4.61, 4.44 and 5.17 ppm which were designated **A, B**, **C**, **D**, and **E**, respectively. The signal at 1.3 ppm was probably derived from H-6 of rhamnose. In ^{13}C NMR spectrum (Fig. 2 and Table 1), residues **C** and **D** overlapped at 102 ppm while residues **A**, **B** and **E** overlapped at 98 ppm (Fig. 5). All the ^{1}H and ^{13}C signals were assigned using homonuclear correlation spectrum (COSY) (Fig. 3), total correlated spectroscopy (TOCSY) (Fig. 4), heteronuclear multiple-quantum coherence spectroscopy (HMQC) (Fig. 5) and heteronuclear multiple bond correlation spectroscopy (HMBC) (Fig. 6).

The intensive anomeric signals of residue **A** at 5.22 ppm (Fig. 1) and 98 ppm (Fig. 2) corresponded to an α linked residue with a relatively high content in 60P. The proton assignment of residue **A** (From H-1 to H-5: 5.22, 3.58, 3.75, 3.26 and 4.26 ppm) was obtained from COSY spectrum (Fig. 3 and Table 2). This assignment was also supported by the well resolved cross peaks in TOCSY spectrum (Fig. 4, **A** line). The corresponding chemical shifts of ^{13}C, revealed by HMQC spectrum, were 98, 72.1, 73, 83 and 73 ppm for C-1, C-2, C-3, C-4 and C-5, respectively (Fig. 5). This result was further confirmed by the intra-correlation cross peaks of residue **A** in HMBC spectrum, in which the cross peaks of H-1 with C-2, C-2 with

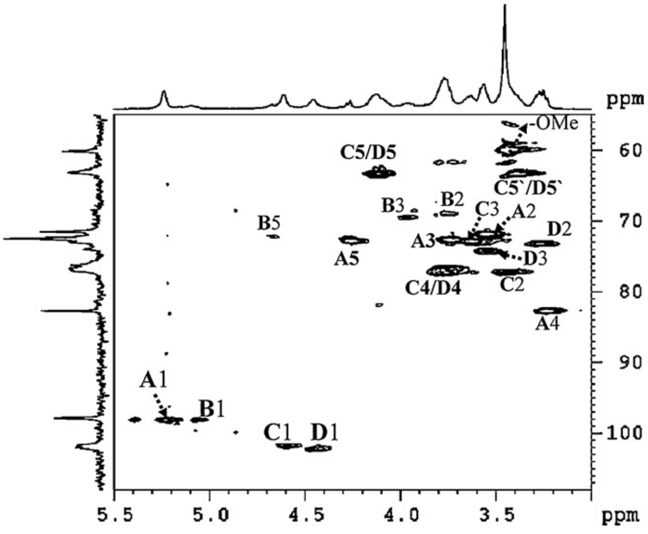


Fig. 5. A part of the HMQC spectrum of polysaccharide 60P isolated from ASK (60 °C in D_2O).

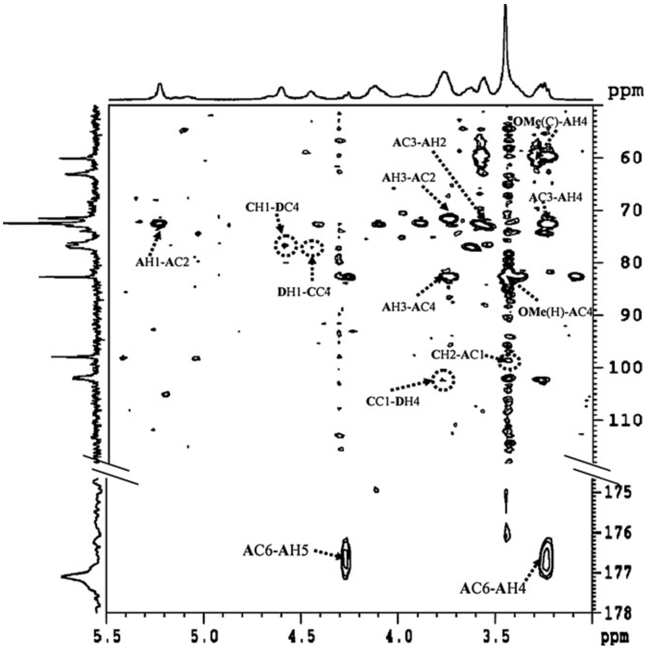


Fig. 6. A part of the HMBC spectrum of polysaccharide 60P isolated from ASK (60 °C in D_2O).

H-3, C-3 with H-2, C-3 with H-4, and H-3 with C-4 were all tagged in Fig. 6. In addition, the chemical shift of C-6 at 177 ppm was also evidenced by the HMBC, in which the cross peaks of C-6/H-5 and C-6/H-4 were clearly observed (Fig. 6). By comparing literature data in combination with all the ^{1}H and ^{13}C chemical shifts, especially C-6 (177 ppm), residue **A** was assigned as terminal-glucuronic acid (Agrawal, 1992; Gutiérrez de, Martínez, Sanabria, de Pinto & Igartuburu, 2005; Hannuksela & Hervé du Penhoat, 2004). It is worth noting that another cross peak with the proton chemical shift of 3.46 ppm was observed on **A** line (residue **A**) of TOCSY spectrum (Fig. 4). This peak was not derived from the sugar ring of residue **A** and was tentatively assigned to the proton of methoxy group. The ^{13}C chemical shift of methoxy group was obtained (60.8 ppm) based on the results of HMQC spectrum. Two cross peaks between methoxy group and residue **A** (–*O*-Me (C) with H-4 of residue **A**; -*O*-Me (H) with C-4 of residue **A**) were observed in HMBC spectrum. The data indicated that *O*-Me group was connected with residue **A** at the *O*-4 position, which could also explain the downfield shifting of C-4 in residue **A** (from 73 to 83 ppm) (Agrawal, 1992). The chemical shifts of residue **A** in 1D and 2D spectra (Table 2) were consistent with the previous results (Agrawal, 1992; An, O'Neill, Albersheim, & Darvill, 1994; Cui, Eskin, Biliaderis, & Marat, 1996; León de Pinto, Martínez, & Rivas, 1994; León de Pinto, Martínez, & Sanabria, 2001; Martínez et al., 2003; Vinod et al., 2008) and were also supported by the methylation analysis results, in which the molar ratio of terminal-glucuronic acid was 22.5% (Table 1).

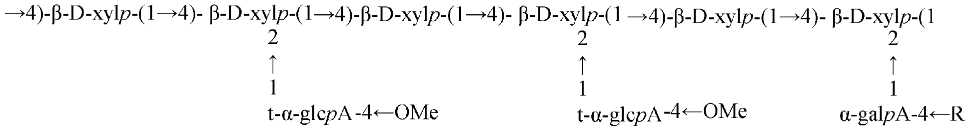
The anomeric signals of residues **C** and **D** centered at 4.61 and 4.44 ppm revealed that both of the residues were β-anomers. The proton assignment of residue **C** was achieved in COSY spectrum (Fig. 3 and Table 2). The chemical shifts of H-1, H-2, H-3, H-4 and H-5/H-5′ were 4.61, 3.42, 3.63, 3.79 and 4.1/3.44 ppm, respectively (Table 2). All the assignments matched perfectly with the TOCSY spectrum (Fig. 4, **C** line) which showed well resolved cross peaks of residue **C** (4.61, 3.42, 3.62, 3.79 and 4.10 ppm). Based on the proton chemical shifts, the ^{13}C chemical shifts of residue **C** were completely assigned as 101.8, 77.2, 73.5, 77.1/73, and 63.2 ppm for C-1 to C-5 in HMQC spectrum (Fig. 5). Results reported by Hannuksela and Hervé du Penhoat (2004) and León de Pinto, Martínez, and Rivas (1994) indicated that the C-4 chemical shift should be 77.1, rather than 73 ppm, which was further verified by the cross peaks marked on HMBC spectrum (Fig. 6). The anomeric ^{1}H and ^{13}C chemical shifts of residue **D** were similar to residue **C**, suggesting that these two spin systems were similar. Combined with TOCSY (Fig. 4, **D** line) and COSY (Fig. 3) spectra, all the proton chemical shifts of residue **D** were assigned as 4.44, 3.28, 3.57, 3.78 and 4.14/3.41 ppm for H-1, H-2, H-3, H-4 and H-5/H-5′, respectively. All the ^{13}C chemical shifts of residue **D** were obtained from HMQC spectrum (Fig. 5). Comparison of proton and ^{13}C chemical shifts with previous data (Agrawal, 1992; Golovchenko, Ovodova, Shashkov, & Ovodov, 2002; Hannuksela & Hervé du Penhoat, 2004; Hoffmann, Geijtenbeek, Kamerling, & Vliegenthart, 1992; León de Pinto, Martínez & Rivas, 1994; Mort, Zheng, Qiu, Nimtz, & Bell-Eunice, 2008) allowed to assign residue **C** to a 2,4-di-*O*-substituted β-xyl*p* and residue **D** to β-xyl*p* substituted at *O*-4 position.

A complete assignment of the signals derived from 4-linked α-D-galacturonic acid (residue **B**) was achieved and shown in Table 2. The α-configuration of residue **B** was established by signals at 5.08 ppm (H-1) and 98 ppm (C-1). The cross peak between the H-1 and H-2 was not observed in the COSY spectrum (Fig. 3) which created the difficulties during assignment. This issue however was addressed by comparing the chemical shifts with literature values (Cui, Eskin, Biliaderis & Marat, 1996; Golovchenko, Ovodova, Shashkov & Ovodov, 2002; Mort, Zheng, Qiu, Nimtz & Bell-Eunice, 2008; Roy et al., 2007) and by examining the cross peaks in HMQC (Fig. 5). This result was also consistent with the GC–MS data in which the molar ratio of 4-Gal*p*A was 5.6% (Table 1).

Based on the results of monosaccharide composition analysis (Guo et al., 2010) and methylation analysis, T-Ara*f*, T-Ara*p* and 3-Ara*f* were present in the 60P fraction. The molar ratios of T-Ara*f* and T-Ara*p* were 1.7% and 1.1% while the exact amount of 3-Ara*f* was not obtained due to the overlap with adjacent peak (Table 1). In NMR spectroscopy, the residue **E** was tentatively assigned to T-β-Ara*f* by comparing the proton chemical shifts (only obtained from TOCSY spectrum in Fig. 4) with literature values (Cardoso, Ferreira, Mafra, Silva, & Coimbra, 2007; Jensen et al., 2010). However, due to weak signals, the full assignments of ^{1}H and ^{13}C of the residue **E** could not be achieved in NMR spectrum; T-Ara*p* and 3-Ara*f* residues were not observed in the NMR spectrum and needs further clarification (Table 2).

Examining the cross-peaks of both anomeric protons and carbons of each sugar residue in HMBC spectrum, both inter- and intra-residual connectivities were evident (Fig. 6). Cross-peaks between H-1 (4.61 ppm) of residue **C** and C-4 (76.7 ppm) of residue **D**; H-1 (4.44 ppm) of residue **D** and C-4 (77.1 ppm) of residue **C**; C-1 (102.5 ppm) of residue **A** and H-2 (3.42 ppm) of residue **C**; H-4 (3.78 ppm) of residue **D** and C-1 (101.8 ppm) of residue **C** were observed, indicating that some of the terminal-α-glucuronic acid were linked to 2,4-α-xyl*p* through 1,2-*O*-glycosidic bonds; 4-β-xyl*p* and 2,4-β-xyl*p* were linked to each other through a 1,4-*O*-glycosidic bond as main chain in the structure.

Based on the results of monosaccharide composition, methylation analysis and NMR spectroscopy, the following structure was proposed for 60P:



$$\begin{array}{cccccc}
\rightarrow 4)\text{-}\beta\text{-D-xyl}p\text{-}(1\rightarrow 4)\text{-} & \beta\text{-D-xyl}p\text{-}(1\rightarrow 4)\text{-} & \beta\text{-D-xyl}p\text{-}(1\rightarrow 4)\text{-} & \beta\text{-D-xyl}p\text{-}(1\rightarrow 4)\text{-} & \beta\text{-D-xyl}p\text{-}(1\rightarrow 4)\text{-} & \beta\text{-D-xyl}p\text{-}(1 \\
 & 2 & & 2 & & 2 \\
 & \uparrow & & \uparrow & & \uparrow \\
 & 1 & & 1 & & 1 \\
 & \text{t-}\alpha\text{-glc}p\text{A-4}\leftarrow\text{OMe} & & \text{t-}\alpha\text{-glc}p\text{A-4}\leftarrow\text{OMe} & & \alpha\text{-gal}p\text{A-4}\leftarrow\text{R}
\end{array}$$

R represents one of the following groups: T-Ara*f*, T-Ara*p*, T-Ara*f* → 3-Ara*f* (1→ and T-Ara*p* → 3-Ara*f* (1→. This structure is very similar to that of polysaccharide from *Cercidium praecox* (R&P) Harms exudate which was reported by León de Pinto, Martínez, and Rivas (1994). However, the structure proposed in the present paper has not been reported for seed polysaccharides.

4. Conclusion

In the present study, a new structure was proposed for the high molecular weight fraction of polysaccharides from *A. sphaerocephala* Krasch Seed. The results of monosaccharide analysis, methylation analysis and 1D & 2D NMR all support a structure that consists of 1,4-linked xyl*p* backbone, branched at every second xyl*p* on the *O*-2 position with mostly T-α-Glc*p*A-4 ← *O*Me, and occasionally 4-α-D-gal*p*A, T-Ara*f*, T-Ara*p* and 3-β-Ara*f*.

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