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Note

Chemical structures of water-soluble polysaccharides from *Rhizoma Panacis Japonici*

Zhiping Huang [a,b], Lina Zhang [a,*]

[a] Department of Chemistry, Wuhan University, Wuhan 430072, China
[b] The 42nd Institute of the Fourth Academy of CASC, Xiangfan 441003, China

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ABSTRACT

Five polysaccharide samples, coded as RPS1, RPS2, RPS3, RPS4, and RPS5, were isolated stepwise from *Rhizoma Panacis Japonici* (RPJ) by using 0.15 M NaCl aqueous solution at 25 °C, boiling water at 120 °C, 0.5 M NaOH/0.01 M $NaBH_4$ at 10 °C, 1.0 M NaOH/0.02 M $NaBH_4$ at 10 °C, and 19 M HCOOH at 4 °C, respectively. The yields were 0.39%, 1.08%, 2.41%, 0.32%, and 0.04% for RPS1 to RPS5, respectively. The chemical structures of the polysaccharides were highly branched α-(1→4)-D-glucan heteropolysaccharides and the values of degree of branch (*DB*) were in the range of 35–45% for RPS1 to RPS5. All of the polysaccharides were water soluble, and their solubility decreased from RPS1 to RPS5. The weight average molecular mass were 3.5×10^4, 1.47×10^5, 1.24×10^6, 9.26×10^5, and 1.36×10^6 for RPS1 to RPS5, respectively.

Rhizoma Panacis Japonici (RPJ), which grows in Chinese midland mountains, is a Chinese traditional medicine. It has been used as drug and as a substitute for *Panax Ginseng* for several hundred years due to its anti-inflammatory, antitussive, expectorant, gastrointestinal, analgesic, muscle contraction, hypnotic, and sedative efficacies.[1,2] Polysaccharides are diversified in the nature, they play important roles in living organisms in almost every aspect of physiology events similar to deoxyribonucleic acid and protein, and they are also used as antitumor and immunity agents.[3–13] Moreover, the structures and conformations of polysaccharides are responsible for their behaviors in solution and their interactions with other molecules or cells.[14–18] It has been reported that the chemical structure of polysaccharides, namely sugar composition and position of glycosidic linkages, can be analyzed by gas chromatography (GC) and/or mass spectrometry (GC/MS),[17] high performance liquid chromatography (HPLC) with tandem mass spectrometry (MS/MS),[18] as well as by time of flight mass spectrometry (TOFMS).[18–21] The anomeric configuration (i.e., α or β style) of each sugar residue can be determined by nuclear magnetic resonance (NMR) spectroscopy[21–28] and Fourier transform infrared (FTIR) spectroscopy.[29,30]

We isolated five polysaccharides (coded as RPS1, RPS2, RPS3, RPS4, and RPS5) from RPJ in different media and confirmed that they were soluble in water. The solubility of the polysaccharides was in the range of 1–10 g/100 g water which decreased from RPS1 to RPS5 with the isolation progress. The size exclusion chromatograms (SECs) of these samples exhibited single peak and the weight average molecular mass, determined by laser light scattering (LLS, Dawn EOS, Wyatt Technology Corporation, USA), were 3.5×10^4, 1.47×10^5, 1.24×10^6, 9.26×10^5, and 1.36×10^6 for RPS1 to RPS5, respectively.

Ultraviolet absorption spectrum (UV-160, Shimadzu, Japan, not shown) of the five samples showed a strong diagnostic peak at 200 nm for polysaccharide and absence of peak at 280 nm and 600 nm for protein and pigment, which demonstrated that the five samples were pure polysaccharides. In FTIR spectra of all samples (not shown), the broad peak at about 3410 cm^{-1} was assigned to the stretching vibration of hydroxyl, and the signals at 1420 cm^{-1} and 1240 cm^{-1} were derived from the associated and free bending vibration of hydroxyl. The elemental analysis of all samples revealed that there were mainly carbon (C), hydrogen (H) and oxygen (O), and the molar ratios of C/H/O were around 1:2:1, which corresponded to those of typical neutral polysaccharides.

The most intense peak in GC analysis (not shown) at retention time of 20.7 min was identified to be glucose (Glc) by comparing with the retention times of standard monosaccharides, and the relative weaker peaks were identified to be galactose (Gal), arabinose (Ara), xylose (Xyl), and mannose (Man). The glucose content was about 45% for RPS1, 90% for RPS2 and RPS3, 60% for RPS4 and RPS5 by weight (Table 1). The fact that the molar ratios of Glc/Gal were about 1:1, 19:1, 18:1, 3:1, 2:1 for RPS1 to RPS5 suggested that RPS2 and RPS3 were relative pure glucan. Figure 1 shows the total ion current (TIC) chromatograms of GC/MS from methylation

* Corresponding author. Tel.: +86 27 87219274; fax: +86 27 68754067.
E-mail address: lnzhang@public.wh.hb.cn (L. Zhang).

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Table 1
GC results of the hydrolysis of the polysaccharides

Retention time (min) Ascription	15.1 Ara (%)	15.3 Xyl (%)	20.7 Man (%)	20.8 Glu (%)	21.2 Gal (%)	Molar ratio Glc/Gal
RPS1	17.6	—	4.2	43.1	35.0	1
RPS2	4.9	—	—	90.3	4.8	19
RPS3	2.5	2.8	—	89.7	4.9	18
RPS4	6.2	14.9	—	60.6	18.2	3
RPS5	13.6	—	—	60.4	26.0	2

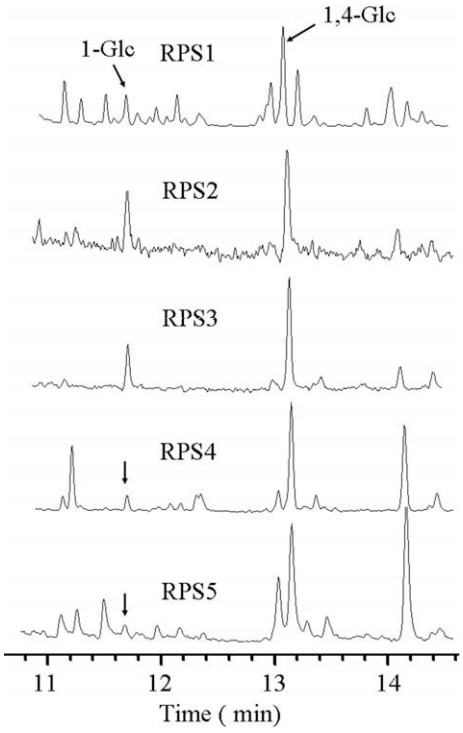


Figure 1. GC/MS TIC chromatograms of the polysaccharides methylation analyses.

analyses of the five polysaccharides. The most intense peak at retention time 13.2 min was ascribed to 2,3,6-tri-*O*-methyl-D-glucose, indicating (1→4)-Glc linkage, whereas peak at 11.7 min was assigned to 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol, indicating 1-Glc end residue. The ascription of other small peaks indicated that there were additional 1,4,6-Glc, (1→3)-Gal, (1→6)-Gal, (1→4)-Ara, (1→3)-Man, and/or (1→4)-Xyl linkages in the polysaccharides. GC/MS results of the methylation analyses of the polysaccharides are summarized in Table 2. From Table 2, it can be found that there was one branch glucose residue per three glucose residues for RPS1, per four glucose residues for RPS2, per six glucose residues for RPS3, and per two glucose residues for RPS4 and RPS5 by the ratio of total glucose to branch glucose. The branch position was O-6 deduced from the existence of 1,4,6-Glc linkages. The molar ratios of glucose determined by GC/MS were in good agreement with the results obtained from GC. Therefore, the glucose was found in all studied samples with (1→4) linkage and branches. The degree of branch (*DB*) could be calculated from the following equation:[31,32]

$$DB = \frac{N_T + N_B}{N_T + N_B + N_L} \quad (1)$$

where, N_T, N_B, and N_L are the numbers of the terminal residues, branch residues, and linear residues, respectively. By using the data from GC/MS, *DB* was calculated to be around 35% for RPS1 to RPS3, 42% for RPS4, and 45% for RPS5, respectively. Thus, the *DB* of the polysaccharides increased with the isolation progress.

In FTIR spectra of RPS1 and RPS5, an absorption peak near 893 cm^{-1} indicated β-style configuration, whereas absorptions at 930 and 850 cm^{-1} in those of RPS2 and RPS3 were assigned to α-configuration. The peaks at 900 and 850 cm^{-1} in FTIR spectra of RPS4 revealed the co-existence of α- and β-configurations.[27] In addition to the main component glucose and small amount of galactose and arabinose in all samples, there were a few mannoses in RPS1 and RPS5, and some xyloses in RPS3 and RPS4, it suggested that the glucose might be in α-configuration, and galactose, arabinose, mannose as well as xylose might be in β-configuration.

The ^{13}C NMR spectra of the five polysaccharides are shown in Figure 2. There were only six strong chemical shifts (δ) at 100.0, 71.7, 73.5, 77.3, 71.5, and 60.7, assigned to C-1, C-2, C-3, C-4, C-5, and C-6 of α-(1→4)-D-glucan, respectively.[18,22,24] The signals at δ 84.0 and 81.5 (only in RPS1, RPS5 as minor peaks) in non-anomeric area indicated a furanoside, whereas the chemical shifts of other peaks in non-anomeric area were lower than δ 80, and demonstrated that other sugar residues existed as pyranoid. The signals at δ 104.5, 81.5 77.3, 84.1 corresponded to C-1, C-2, C-3, C-4 of α-L-arabinofuranose residues,[17] and the signals at δ 107.7, 84.1, 76.8, 81.5, 71.5 corresponded to β-galactanfuranose.[26] The ^1H NMR spectra of the five polysaccharides are shown in Figure 3. A single signal for anomeric proton at δ 5.2 confirmed that the polysaccharides contained mainly one kind of sugar residue assigned to α anomer. The ^{13}C and ^1H NMR results were in good agreement with those obtained from GC and GC/MS as well as from FTIR. Signals at δ 69.6 and 71.0 were attributed to O-substituted C-6 and C-5 of the branched units. The fact that C-2 (δ 71.7) and C-5 (δ 71.5) signals of all samples were almost superimposed suggested that the chemical surroundings of C-2 and C-5 were similar, and demonstrated that the (1→4) linkage existed in the polysaccharides. The peaks in the ^1H NMR spectra broadened and combined to give single peak from RPS1 to RPS5, the results of the increase in molecular mass and the decrease in solubility of the polysaccharides in water, suggested an increase of inter- and intra-molecular hydrogen bond.

The two-dimensional (2D) NMR spectra, namely correlated spectroscopy (COSY) and heteronuclear single quantum coherence (HSQC) of RPS1 and RPS2 are shown in Figure 4. The fact that there were no hydroxyl groups on C-1 and C-4 further confirmed that the

Table 2
GC/MS results of the methylation analyses of the polysaccharides

Retention time (min) Linkage	13.2 1→4-Glc (%)	11.7 1-Glc (%)	13.0 Gal (%)	11.1 Ara (%)	11.2 Xyl (%)	11.3 Man (%)	Total Glc (%)	*DB* (%)	Molar ratio Total Glc/branch Glc
RPS1	24.9	7.1	37.2	10.2	—	5.0	47.6	34.5	3
RPS2	57.2	18.4	3.7	4.1	—	—	92.2	35.9	4
RPS3	56.5	20.9	11.7	—	—	—	88.3	36.2	6
RPS4	32.2	3.9	17.7	2.9	15.9	—	63.4	42.4	2
RPS5	28.1	2.2	17.9	3.5	—	5.5	73.1	45.0	2

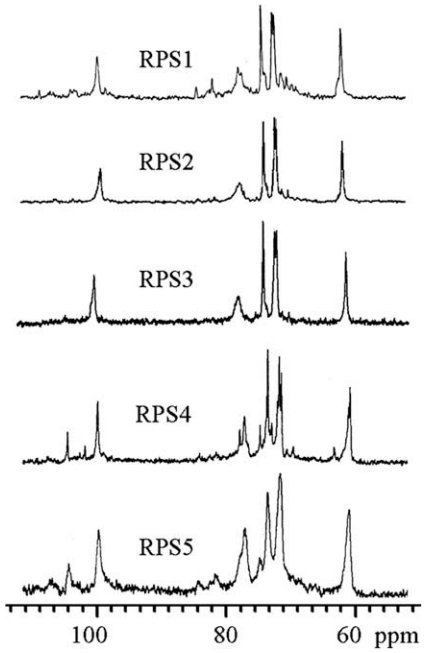


Figure 2. ^{13}C NMR spectra of the polysaccharides (D_2O, 600 MHz).

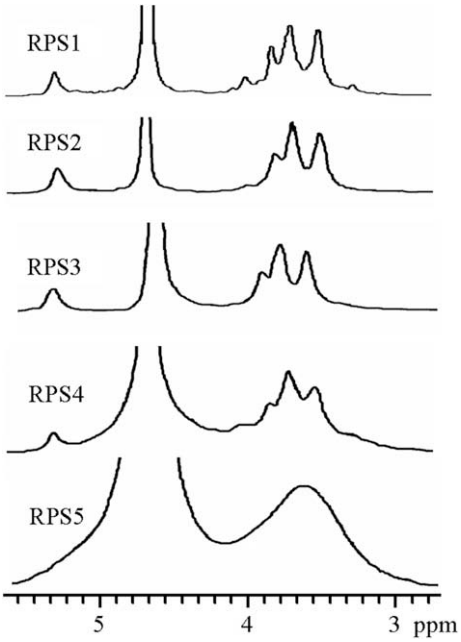
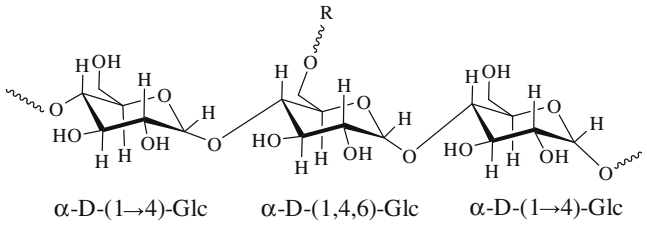


Figure 3. ^{1}H NMR spectra of the polysaccharides (D_2O, 600 MHz).

(1→4) linkage existed in the polysaccharides. The proton correlated intensity on C-6 and O-6 was smaller in RPS1 than in RPS2, indicating more O-substituted on O-6 in RPS1. The ^{13}C and ^{1}H NMR chemical shifts of all samples are listed out in Table 3. In view of the above results, the chemical structure of the polysaccharides is schematically illustrated as following.



R = Gal, Ara, Xyl and / or Man

1. Experimental

1.1. Materials

Rhizoma Panacis Japonici (RPJ) was cultivated in Enshi, Hubei Province, China. Standard monosaccharides, that is, D-glucose, D-galactose, D-arabinose, D-xylose, and D-mannose were purchased from Sigma Chemical (USA). All other chemical reagents were of analytical grade and commercially available and they were used without further treatment.

1.2. Preparation of polysaccharide samples

Dried RPJ was cut into pieces and defatted with ethyl acetate followed by acetone. Polysaccharides were extracted from the defatted RPJ stepwise by using 0.15 M NaCl aqueous solution at 25 °C for 24 h, boiling water at 120 °C for half an hour, 0.5 M NaOH/0.01 M $NaBH_4$ at 10 °C for 5 h, 1.0 M NaOH/0.02 M $NaBH_4$ at 10 °C for 5 h, 19 M HCOOH at 4 °C for 1 h, and each process was repeated three times. The extracting mixtures were centrifuged at 7000 rpm. The supernate from hot water extract was preserved in refrigerator and thawed out as well as centrifuged to remove the metamorphic starch powder. Those supernates from base and acid extracts were neutralized. Then all the supernates were deproteinized by the Savage method, discolored by H_2O_2, condensed by rotatory evaporator, and dialyzed against tap and distilled water for 4 days, respectively. The resulting supernates were lyophilized to obtain five polysaccharide samples coded as RPS1, RPS2, RPS3, RPS4, and RPS5. Saponins in RPS1 were removed by flushing it with methanol in a Soxhlet extractor. The resulting RPS1 was white powder, RPS2 to RPS5 were white scales, and the yields were 0.39%, 1.08%, 2.41%, 0.32%, and 0.04% for RPS1 to RPS5, respectively.

1.3. Characterization

GC/MS was carried on a GCT system (Macro-Mass, Waters, USA) with a capillary GC column (HP-5MS, 30 m × 0.32 mm ID × 0.25 μm, Agilent, USA) using He as carrier gas. The oven temperature was set to increase from 180 °C to 220 °C at a rate of 4 °C/min and the detector temperature was set at 250 °C. The polysaccharide samples were first methylated by CH_3I, then extracted by $CHCl_3$, and were washed with distilled water to remove the unmethylated water-soluble polysaccharide. The permethylated polysaccharide was hydrolyzed in 12 M H_2SO_4 at 20 °C for 1 h, followed by 2 M H_2SO_4 at 90 °C for 2 h, and then acetylated with Ac_2O in pyridine at 90 °C for 3 h. GC was carried out on a GC system (Agilent 6890N, Agilent, USA) equipped with a flame ionization detector at chromatographic condition that was similar to that of GC/MS, and the samples were directly hydrolyzed and acetylated. The standard monosaccharides were also acetylated.

The ^{13}C, ^{1}H and 2D NMR spectra of the polysaccharides were measured on a NMR spectrometer (Inova-600, Varian, USA) in D_2O at 25 °C. Chemical shifts were given relative to external Me_4Si (0 ppm). FTIR spectra were recorded on a FTIR spectrometer (Equinox 55, Bruker, Germany). The samples were ground with KBr at a ratio of 1:20 and pressed into a thin pellet for FTIR analysis. The elemental analysis was carried out by an automatic element analyzer (Elementar, Germany).

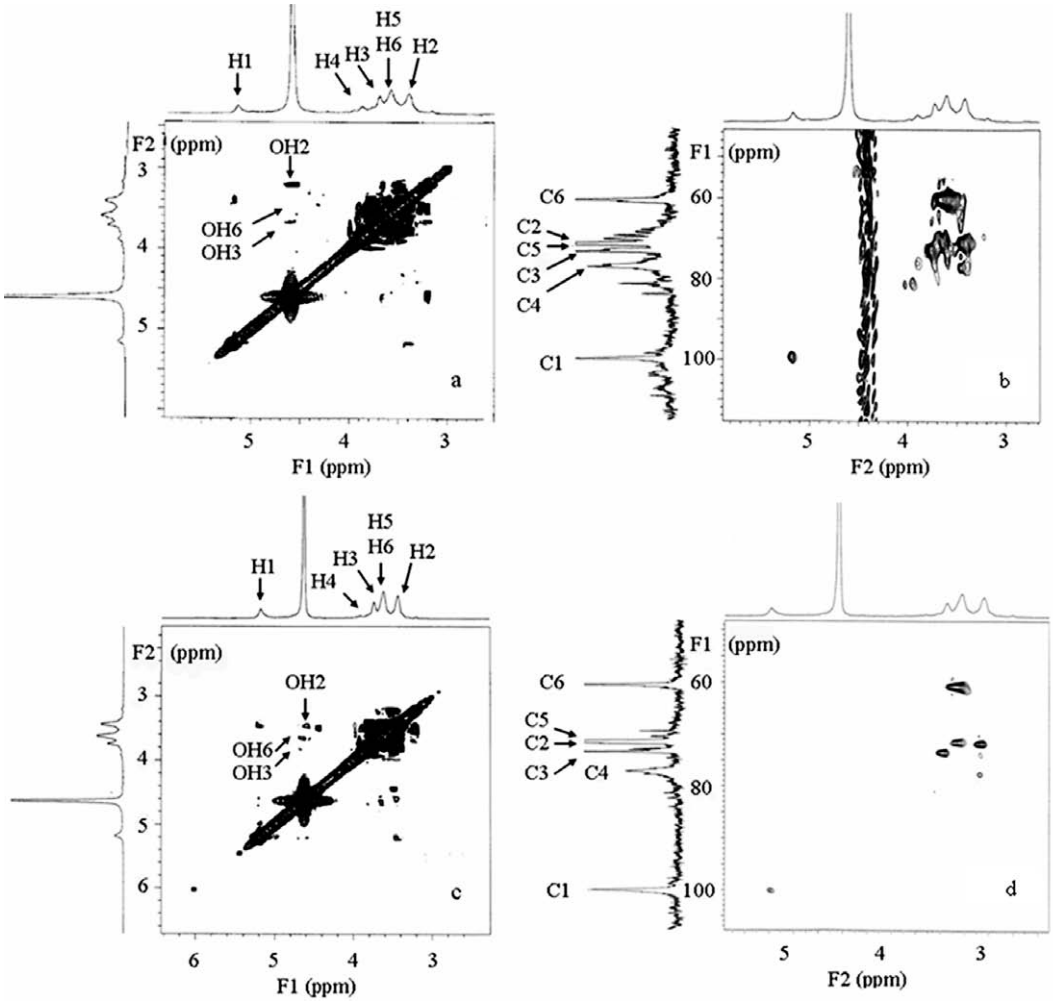


Figure 4. 2D NMR spectra of the polysaccharides: (a) COSY of RPS1, (b) HSQC of RPS1, (c) COSY of RPS2, and (d) HSQC of RPS2.

Table 3
Chemical shift (δ, ppm) data of ^{13}C and ^{1}H NMR for the polysaccharides

Sample	C-1/H-1	C-2/H-2	C-3/H-3	C-4/H-4	C-5/H-5	C-6/H-6
RPS1	100.0/5.2	71.5/3.5	73.5/3.8	77.3/3.9	71.7/3.7	60.7/3.7
RPS2	100.1/5.2	71.7/3.5	73.5/3.8	77.3/3.9	71.4/3.7	60.6/3.7
RPS3	100.2/5.3	71.6/3.6	73.6/3.8	77.5/3.9	71.8/3.8	60.7/3.8
RPS4	99.9/5.3	71.8/3.5	73.6/3.8	77.2	71.5	60.7/3.7
RPS5	99.8	71.6	73.5	77.0	71.6	60.7

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