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Structural investigation of two neutral polysaccharides isolated from rhizome of *Polygonatum sibiricum*

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Abstract

Two neutral polysaccharides, PSW-1a and PSW-1b-2, were isolated from the hot-water extract of *Polygonatum sibiricum* roots, using ethanol precipitation, anion-exchange chromatography, Fehling reagent complexing, and gel permeation chromatography. Their structures were investigated using composition and linkage analyses, periodate oxidation and Smith degradation, partial acid hydrolysis, and NMR spectroscopic methods. PSW-1b-2 was characterized as a branched homogalactan, containing a 1,4-linked β-D-galactopyranosyl backbone with one β-D-galactopyranosyl stub substituted at O-6 of every 7th backbone residue, whereas PSW-1a as a highly branched galactomannan, possessing a 1,4-linked β-D-mannopyranosyl backbone, with a β-D-galactopyranosyl stub attached to O-6 of every 9th mannosyl residue.

Keywords: Galactan; Galactomannan; Polysaccharide; *Polygonatum sibiricum*

1. Introduction

Polygonatum sibiricum Redoute is a folk medicine widely used in China, Korea and Japan. The plant, belonging to the family of *Liliaceae*, is distributed mainly in the northeast of China, and its dried root or stem, after processing, is used in traditional Chinese medicine. The plants of the same genus, *P. kingianum*, *P. cyrtonema*, have also been used as the substitutes of *P. sibiricum*. In traditional Chinese medicine, *P. sibiricum* is used mainly for the treatment of cough, dizziness and lung trouble. Pharmacological studies indicate that *P. sibiricum* may stimulate immune system, decrease blood glucose and lipid levels, and prevent aging (Zheng, Dong, & She, 1998). The reported chemical constituents of *P. sibiricum* include steroid saponins (Son & Du, 1990), flavones (Chopin, Dellamonica, & Besson, 1977), alkaloids (Sun, Li, & Wang, 2005), lignins (Sun & Li, 2001), amino acids (Wang, Song, & Jin, 2001), and carbohydrates (Barbakadze, Kemertelidze, Dekanosidze, & Usov, 1993; Yang & Yu, 1980). The polysaccharides isolated from *P. sibiricum* have been reported to show immunomodulatory, anti-aging and antiviral activities (Gu, Meng, & Pu, 2003; Shi, Meng, & Li, 1999), and they may be the major bioactive components of the crude drug. During our studies on the bioactive polysaccharides of *P. sibiricum*, we isolated six homogeneous polysaccharides from hot-water extract, and two polysaccharides from alkali extract, respectively. In this paper we describe the isolation and structural investigation of a homogalactan PSW-1b-2 and a galactomannan PSW-1a, which were first reported in *P. sibiricum*.

2. Experimental

2.1. Materials

The crude drug of the rhizome of *P. sibiricum*, which had been processed by heating in water vapor (100 °C) for 12 h, dried, and cut into small slices (4 $cm^2 \times 3$ mm

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thick), was purchased from local market (Shanghai Xuhui Herb Slices Co. Ltd). The monosaccharide standards were purchased from Fluka, DEAE-cellulose 52 was from Whatman Co. Sephacryl S-300, Sephadex G-75 and G-150 were from Amersham Biosciences. Bio-Gel P2 was from Bio-Rad Co. Membra-CEL™ dialysis tube (MW cut-off 3500) was purchased from Sino-American Biotechnology Co. All other chemicals were of reagent grade unless otherwise claimed.

2.2. General methods

All evaporations were carried out under depressed pressure at below 45 °C. Optical rotation was recorded on a WZZ-1S polarimeter (Shanghai Physical Optics Instrument Co.). GLC was performed on a Shimadzu GC-14B instrument equipped with a 3% OV-225 packed glass column (2.1 m × 3.0 mm) and an FID detector. The column temperatures for sugar composition and methylation analysis were 210 and 190 °C, respectively. TLC was performed on a precoated Pei-cellulose plate (E. Merck), with ethyl acetate–pyridine–water–acetic acid (5:5:3:1) as the developing solvent, and visualized by spraying with *o*-phthalic acid–aniline reagent and heating. The homogeneity of polysaccharides was detected using high performance gel permeation chromatography (HPGPC) method (Duan, Wang, Dong, Fang, & Li, 2003; Wei & Fang, 1989). Neutral carbohydrate was determined with sulfuric acid–phenol method (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956), with D-glucose as standard. Uronic acid was determined by *m*-hydroxyl diphenyl method (Blumencrantz & Asboe-Hansen, 1973), with D-glucuronic acid as standard. Protein was determined by Lowry method (Bensadoun & Weinstein, 1976), with bovine serum albumin as standard.

2.3. Isolation and purification of polysaccharides

To remove lipid the dried crude drug of *P. sibiricum* (3 kg) was soaked in 95% ethanol at rt for 1 week with intermittent stirring, and the extraction was repeated thrice. The residue was air-dried (yield 2.25 kg) and extracted with boiling water five times, each for 4 h. The extract was concentrated and dialyzed. The retentate was concentrated, centrifuged, and to the supernatant was added 3 vols of 95% ethanol. The precipitate was washed with absolute ethanol and acetone, then dried in vacuum, yielding the water-extractable crude polysaccharide, PSW (133 g).

The residue was extracted twice with 1 M NaOH (10 L) at 4 °C for 3 h. The extract was combined, neutralized with 2 M HCl, and dialyzed. The retentate was centrifuged to remove the insoluble, and the supernatant was concentrated and precipitated with 4 vol of 95% ethanol. After dehydration with absolute ethanol and acetone the precipitate was dried in vacuum, giving the alkali-extractable crude polysaccharide, PSB (6.5 g).

To remove the contaminating protein, PSW (40 g) was treated with 15% (w/v) trichloroacetic acid (TCA) at 4 °C for 2 h. After centrifugation the supernatant was neutralized with 10% NaOH (w/v) at 4 °C, then dialyzed against running water. The retentate was freeze-dried, yielding PSW' (33 g). 20.5 g of PSW' dissolved in distilled water (200 mL) was applied to a column of DEAE-cellulose (Cl^-, 80 cm × 6 cm), which was eluted stepwise with water, 0.2, 0.5, 0.8 M NaCl, and 0.1 M NaOH, giving PSW-1 (7.24 g, 35.3% of PSW' mass), PSW-2 (4.51 g, 22.0%), PSW-3 (5.09 g, 24.8%), PSW-4 (1.73 g, 8.4%), and PSW-5 (0.44 g, 2.1%), respectively. In the same manner PSB (6.0 g) was fractionated, and the column was eluted stepwise with distilled water, 0.1, 0.2, and 0.5 M NaCl, yielding four fractions, PSB-1 (0.50 g, 8.3% of PSB), PSB-2 (0.43 g, 7.2%), PSB-3 (0.21 g, 3.5%), and PSB-4 (0.43 g, 7.2%).

For PSW-1 (0.35 g) dissolved in water (35 mL), Fehling reagent (5 mL) was added (Jones, 1965). The mixture was stirred for 4 h, and centrifuged. The supernatant was neutralized with 25% (w/v) acetic acid to pH 7.0, and dialyzed. The retentate was concentrated and precipitated with 3 vol of ethanol, giving PSW-1b (210 mg). The precipitate was treated with 5% AcOH in ethanol (v/v) at 0 °C (1 min), and filtered. The precipitate was dehydrated with ethanol and acetone, and dried in vacuum, giving PSW-1a (52 mg). PSW-1b was purified on a column of Sephadex G-75 (2.6 cm × 90 cm), with distilled water as eluent, giving PSW-1b-2. PSW-1a is not soluble in water. Its homogeneity was estimated by chromatography on a column of Sephadex G-75, with 0.5 M NaOH as the solvent and detected with phenol–sulfuric acid method.

PSW-2, PSW-3, PSW-4, and PSW-5 were separated on a column of Sephacryl S-300, giving PSW-2A-1, PSW-3A, PSW-4A, and PSW-5B, respectively. The alkali-extracted fractions, PSB-1 and PSB-2, were purified by repeated chromatography on a column of Sephadex G-150, giving PSB-1B and PSB-2A, respectively. 0.2 M NaCl was used as the eluent, and the fractions were monitored by phenol–sulfuric acid method.

2.4. Glycosyl analyses

Polysaccharide (2 mg) was hydrolyzed in 2 mL of 2 M trifluoroacetic acid (TFA) at 110 °C for 1.5 h. TFA was removed by evaporation at reduced pressure with the addition of methanol. The hydrolysate was analyzed by TLC or by GLC after converted into the corresponding alditol acetates (Dong, Ding, & Fang, 1998). For the acidic polysaccharide another part of it was carboxyl-reduced with $NaBH_4$ (Taylor & Conrad, 1972), and then hydrolyzed and analyzed by GLC as described above. The sugar composition was quantitated by comparison of the results from native and carboxyl-reduced polysaccharides.

2.5. Methylation analysis

The vacuum-dried polysaccharide (5 mg) was methylated thrice with methyl iodide and powdered sodium hydroxide in dimethyl sulfoxide as described by Needs

(Needs & Selvendran, 1993). The completeness of methylation was confirmed by the disappearance of the hydroxyl absorption in IR (Nujol). The partially methylated alditol acetates were prepared and analyzed by GC–MS (Dong, Jia, & Fang, 2006).

2.6. Periodate oxidation

Polysaccharide (25 mg) was dissolved in 0.015 M $NaIO_4$ (20 mL), and kept at 4 °C in dark. The reaction was monitored daily with spectrophotometric method at 223 nm (Dixon & Lipkin, 1954). After 72 h, the excessive $NaIO_4$ was decomposed with ethylene glycol (0.1 mL). The $NaIO_4$ consumption was calculated according to the decrease of absorbance (A_{223}) and the formic acid production was determined by titration with 0.01 N NaOH. The reaction mixture was dialyzed against distilled water, and the retentate was reduced with $NaBH_4$ overnight. After neutralization and dialysis the retentate was freeze-dried, and analyzed for sugar composition.

2.7. ^{13}C and ^{1}H NMR spectra

Forty-five milligrams of the polysaccharide sample was dissolved in D_2O (99.8% D, 0.5 mL), freeze-dried, and redissolved in D_2O (0.5 mL). The ^{13}C NMR (100 MHz) and ^{1}H NMR (400 MHz) spectra were measured in a Φ5 mm NMR tube at rt with a Brucker AM-400 NMR spectrometer with a dual probe in the FT mode. All the chemical shifts were in relative to Me_4Si, with acetone as internal reference (δ31.50 ppm) for ^{13}C NMR and HDO (δ4.85 ppm) for ^{1}H NMR. The DEPT experiments were done using a polarization-transfer pulse of 135°.

3. Results and discussion

Two crude polysaccharides were obtained by hot-water extraction and alkaline extraction from the crude drug (3 kg) of *P. sibiricum*, designated PSW (133 g, 4.4% of the crude drug mass) and PSB (6.5 g, 0.2% of the crude drug mass), respectively. Water extraction gave a much higher yield than alkaline extraction. PSW contains 24.6% (w/w) protein so it was treated with 15% (w/v) TCA at 4 °C for 2 h to remove protein. The result showed PSW' obtained after TCA treatment remained to contain some protein. Moreover PSW' showed slight but significant decrease in the content of rhamnose, arabinose, indicating 15% TCA lead to the partial removal of these acid labile residues although some measures (low temperature and short time) have been observed.

The polysaccharide fractions, obtained from anion-exchange chromatography and gel permeation chromatography, were detected by HPGPC for their homogeneity. The homogeneous polysaccharide fractions, illustrated by a symmetrical peak in HPGPC, were analyzed for molecular weights, specific rotations, sugar composition, uronic acid and protein contents, and the results were given in Table 1.

PSW-1, the water-eluted fraction, is composed mainly of Man and Gal, in the ratio of *ca* 1.0:4.4, different from typical galactomannan in its high galactose content, indicating PSW-1 may be a mixture of galactan and mannan. Fehling reagent was used hereby to selectively precipitate mannan by complexing with copper. In this way PSW-1 was separated into the precipitate (PSW-1a) and supernatant (PSW-1b-2) fractions. Monosaccharide analyses and uronic acid determination showed they were both neutral polysaccharides. PSW-1a was hardly soluble in water, as other (1 → 4)-β-D-mannan. It seems that the association between PSW-1a and PSW-1b-2 significantly improve its water-solubility. Acetyl groups have often been reported for (1 → 4)-β-D-mannan or galactomannan. However the Fehling reagent complexing was performed under strong alkali condition, which may lead to the deacetylation and thus change the solubility. Therefore we are not sure if PSW-1a or PSW-1b-2 is acetylated in its native status. Other PSW fractions eluted with different concentrations of NaCl and 0.1 M NaOH are all acidic polysaccharides. All these fractions showed a high content of galactose and the absence of glucose except for PSW-5B, and this is probably characteristic for the polysaccharides of *P. sibiricum*.

According to the glycosyl composition, PSW-1a is a galactomannan and PSW-1b-2 a homogalactan. PSW-2A-1 and PSW-3A-1 showed a typical composition of pectic polysaccharides. PSW-4A and PSW-5B, which were eluted at high NaCl concentration, both showed a high content of protein, indicating the occurrence of coelution of protein with polysaccharides or the polysaccharide–protein complex nature of these two fractions. In contrast the two alkali-extractable fractions, PSB-1B and PSB-2A, showed different sugar compositions, featured by the presence of xylose, indicating a heteroxylan for PSB-2A and a neutral heteropolysaccharide for PSB-1B. In this paper, only the structural investigation of PSW-1a and PSW-1b-2 was described.

3.1. The structural investigation of PSW-1b-2

HPGPC showed that PSW-1b-2 is a homogeneous fraction. It was found to contain only galactose as the component sugar by GLC as alditol acetates. IR and *m*-hydroxyl diphenyl method indicated the absence of uronic acid, and so PSW-1b-2 is a neutral homogalactan. The absorption at 889.0 cm^{-1} implicated β anomeric configuration for the galactose residues. Methylation analysis showed that the polysaccharide consists of 1,4-, 1,4,6-linked and nonreducing terminal galactosyl residues, in the ratio of 1.1:5.7:1.0, indicating a branched 1,4-linked galactopyranan.

Two periodate oxidation procedures were performed sequentially for PSW-1b-2 to achieve complete oxidation. The first oxidation (72 h) showed that 0.84 mol of $NaIO_4$ was consumed and 0.12 mol of formic acid produced for 1 mol of galactose residues. In comparison with the

Table 1
The molecular weight, specific rotation, and chemical composition of the polysaccharides isolated from *Polygonatum sibiricum*

Fractions	PSW	PSW′	PSW-1a	PSW-1b-2	PSW-2A-1	PSW-3A-1	PSW-4A	PSW-5B	PSB	PSB-1B	PSB-2A
MW (kd)	na	na	na	42	360	200	320	180	na	17	16
$[\alpha]_D$	na	na	na	+61.0	+30.9	+92.0	+237.6	+195.9	na	+54.0	−11.6
Carbohydr. (w%)	66.5	78.0	100	100	70.5	50.1	32.1	34.4	58.7	91.6	80.2
Protein (w%)	24.6	11.6	–	–	–	17.0	45.9	71.4	28.8	–	–
UA (w%)	10.2	13.6	–	–	19.6	31.6	22.5	11.6	10.6	–	17.3
Glycosyl composition (mol%)											
Rhamnose	7.1	4.5	–	–	20.2	29.2	19.6	12.3	–	–	–
Arabinose	6.3	4.8	–	–	11.9	10.4	27.5	18.5	15.9	–	16.1
Xylose	2.8	2.9	–	–	–	–	–	–	57.1	17.1	71.0
Mannose	10.3	10.9	88.8	–	–	–	–	–	–	–	–
Galactose	60.3	60.5	11.2	100	51.2	20.8	37.3	42.0	20.8	69.7	–
Glucose	2.3	2.9	–	–	–	–	–	16.0	6.2	13.2	–
Galacturonic acid	na	na	–	–	16.7	39.6	15.7	11.1	na	–	–
Glucuronic acid	na	na	–	–	–	–	–	–	na	–	12.9

[a] na, not available; –, not detected.

calculated value (1.14 mol $NaIO_4$ consumed and 0.14 mol HCOOH produced) based on methylation analysis, the $NaIO_4$ consumption was significantly lower than expected, probably due to the incomplete oxidation of 1,4-linked galactose residues, which arose from the interresidue hemiacetal formation of aldehyde with neighboring hydroxyl group (Ishak & Painter, 1974). GLC analysis of the polyhydroxyl derivative as alditol acetates showed the presence of galactose, indicating some of the galactose residues resisted oxidation. Consequently the polyhydroxyl derivative was further oxidized with $NaIO_4$ and the product was treated in the same manner. GLC showed the new derivative contains erythritol and glycerol in the ratio of 4.6:1 and no galactose was detected, indicating the complete oxidation.

The ^{13}C NMR spectrum of PSW-1b-2 showed two signals in the anomeric region (Fig. 1), at δ107.4 and 106.6 ppm, indicating the β anomeric configuration for all galactose residues. The stronger group of signals corresponded to 1,4- and 1,4,6-linked residues, and the weaker group corresponded to the residues at nonreducing terminals (Table 2). The weak signal at δ73.50 ppm with a reversed peak in the DEPT spectrum could be attributable to the substituted C-6 of 1,4,6-linked residues. A cross peak corresponding to the H-1 (δ4.48 ppm) and the C4 (δ80.72 ppm) of 1,4-linked Gal in HMBC spectrum (Fig. 2) indicated long-range correlation between H-1 of a backbone galactose and C4 of its neighboring 1,4-linked

Table 2
Assignment of ^{13}C NMR signals for PSW-1b-2

Residues	C-1	C-2	C-3	C-4	C-5	C-6
β-Gal*p*(1→	106.56	73.80	75.77	71.10	78.48	64.05
→4)Gal*p*(1→	107.42	74.88	76.36	80.72	77.57	63.80
→4,6)Gal*p*(1→	107.42	74.88	76.36	80.72	77.57	73.50

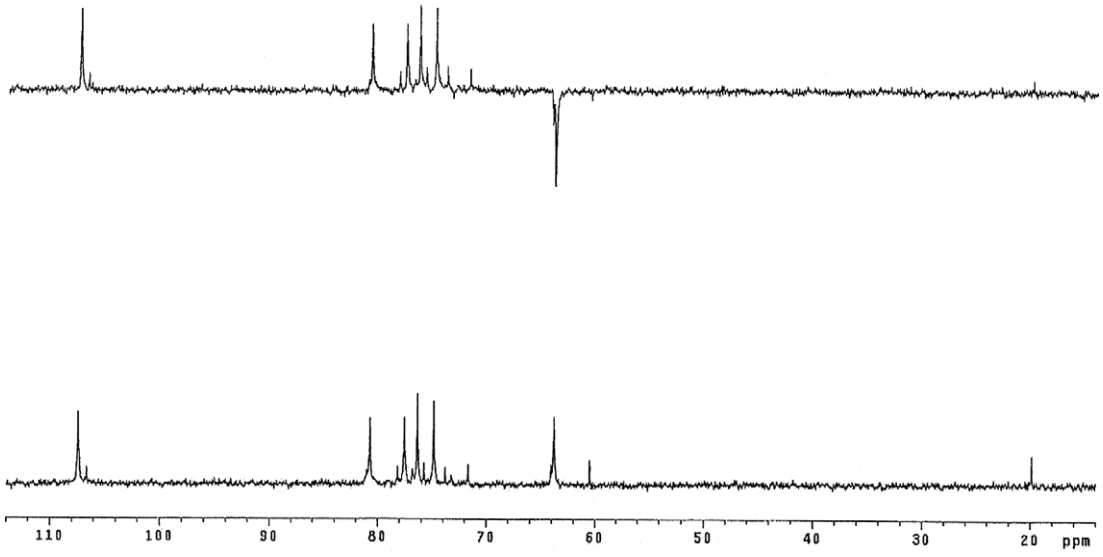


Fig. 1. ^{13}C NMR spectrum of PSW-1b-2.

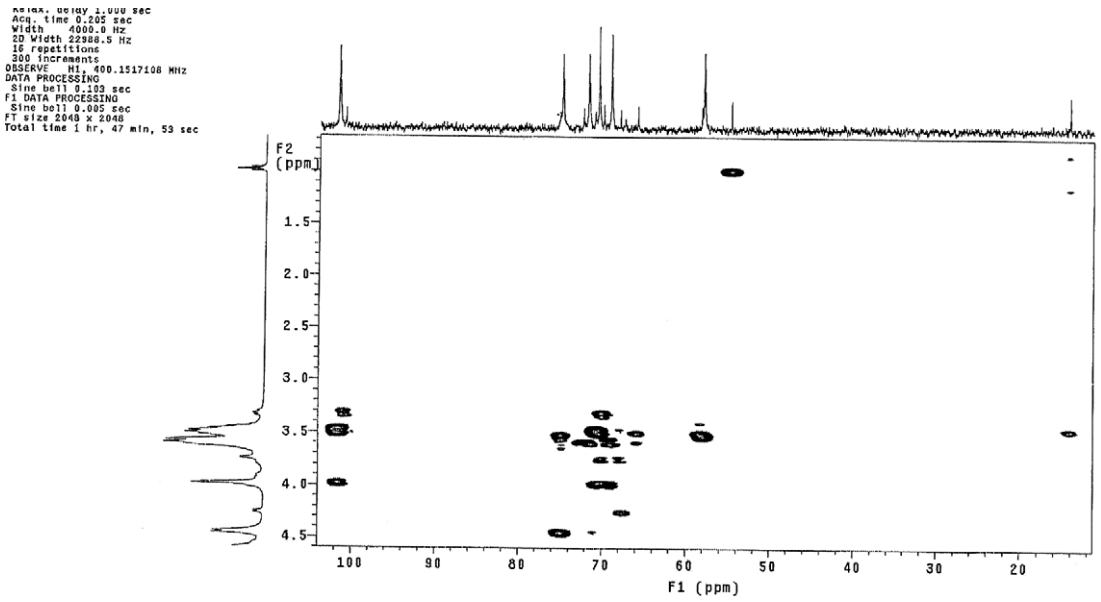


Fig. 2. HMBC spectrum of PSW-1b-2.

galactose residues, in agreement with the β-(1 → 4)-linked backbone structure suggested above. The C1 (δ106.65 ppm) of terminal galactose was correlated to C6 (δ3.45 ppm) of 1,4,6-linked galactose, indicating the terminal galactose is directly attached to O-6 of branching residues, as was corroborated by the correlation between H-1 (δ4.28 ppm) of terminal galactose and C-6 (δ73.50 ppm) of 1,4,6-linked galactose.

Taken together, the results above showed that PSW-1b-2 is a β-D-galactan containing a backbone consisting of 1,4-linked residues, with one nonreducing terminal attached to O-6 for every seventh backbone residue, as shown below.

β-D-Gal*p*-(1
↓
6
→4)-β-D-Gal*p*-(1→[4)-β-D-Gal*p*(1→]$_6$

3.2. The structural investigation of PSW-1a

PSW-1a was not soluble in water so it was dissolved in 0.5 M NaOH and separated on a column of Sephadex G-75 and eluted with 0.5 M NaOH. The eluate was fraction-collected and monitored by carbohydrate content. The chromatographic profile gave a symmetrical peak. After complete hydrolysis TLC analysis showed the absence of uronic acid, indicting PSW-1a is also a neutral polysaccharide. GLC analysis of the alditol acetates showed that PSW-1a contains mannose and galactose in the ratio of 7.9:1.0. Methylation analysis identified terminal Gal, 1,4- and 1,4,6-linked Man, in the ratio of 1.1:8.1:0.6. This result indicated a possible 1,4-linked mannan backbone with one galactose substituted at O-6 of every ninth backbone mannose residue.

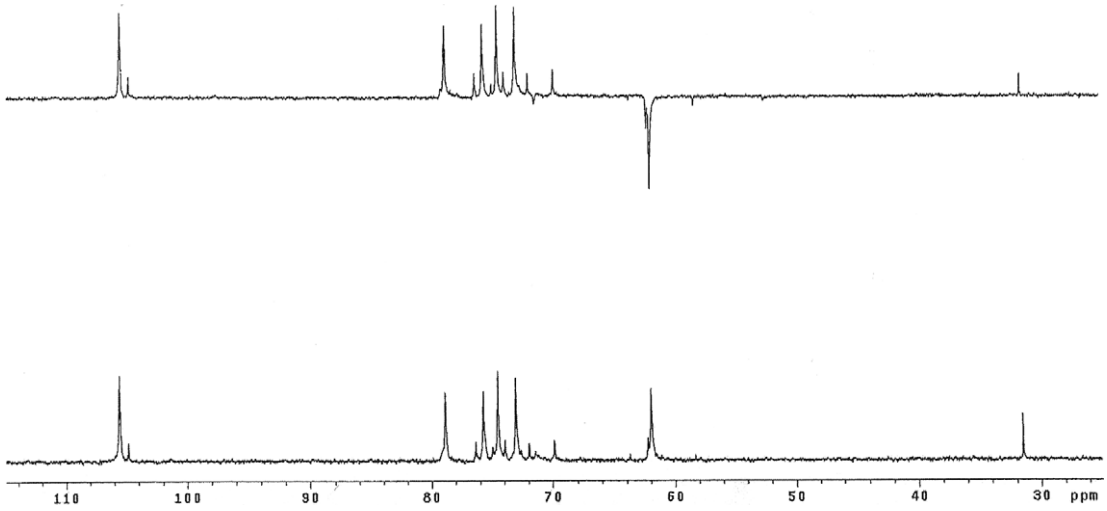
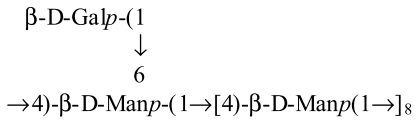


Fig. 3. ^{13}C NMR spectrum of PSW-1a (in 0.5 M NaOD), with Me_2CO as internal reference (δ31.5 ppm).

^{13}C NMR spectrum (Fig. 2) showed two anomeric resonances (δ105.6 and 104.8 ppm), indicating both the Man and the Gal residues are in β-anomeric configuration, which was supported by the H-1 resonances (δ4.65 and 4.45 ppm) in ^{1}H NMR spectrum. The signal at δ78.89 ppm is assigned to C4 of 1,4- and 1,4,6-linked Man. A weak reversed signal in DEPT spectrum at δ69.88 ppm arises from the substituted C-6 of 1,4,6-linked Man (Fig. 3).

The results above indicated that PSW-1a is a branched galactomannan, containing a 1,4-linked β-D-mannosyl backbone, with a single-unit β-D-galactosyl stub attached to O-6 for every the ninth mannosyl residue, as shown below.



The polysaccharide fractions of *Polygonatum* genus have been widely investigated, and various glucomannans with different ratios of Man/Glc and glucofructans were isolated and characterized (Barbakadze et al., 1993; Rakhmanberdyeva & Rakhimov, 1982, 1987). In this communication we isolated and characterized two neutral polysaccharides from *P. sibiricum*, a β-D-galactan and a branched galactomannan, both of which have not been reported for other *Polygonatum* genus plants. It is common that the various species belonging to the same *Polygonatum* genus are reported to contain different polysaccharide components since genetic background, growth condition, and/or season of harvesting all may contribute to such variance. Primary immune tests on *in vitro* T- and B-lymphocyte proliferation models induced by ConA and LPS showed that the crude polysaccharide PSW is effective but both PSW-1a and PSW-1b-2 are inactive, indicating the immunological activity may result from other polysaccharide fractions in PSW.

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