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Structural analysis of water-soluble glucans from the root of *Angelica sinensis* (Oliv.) Diels

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Abstract—Two water-soluble glucans (designated APS-1cI and APS-1cII) were extracted from the roots of *Angelica sinensis* (Oliv.) Diels and further purified by anion-exchange and gel-filtration chromatography. Their molecular weights were determined to be 1.7×10^5 and 3.9×10^4 Da, respectively. The structures of the purified glucans were investigated by a combination of chemical and instrumental analysis, such as methylation analysis, periodate oxidation, GC–MS, as well as FTIR and NMR spectroscopy (^{1}H, ^{13}C, H–H COSY, HSQC, HMBC, TOCSY and NOESY). The data obtained indicated that APS-1cI was a linear α-glucan composed of only (1→6)-α-D-Glc*p*, and APS-1cII had a repeating unit consisting of (1→4)-α-D-Glc*p* and (1→6)-α-D-Glc*p* in a molar ratio of 4:1. Such glucans isolated from *A. sinensis* (Oliv.) Diels have not been previously reported.

Keywords: *Angelica sinensis*; Structure; Polysaccharide; Glucan

1. Introduction

Chinese Danggui is the root of *Angelica sinensis* (Oliv.) Diels (Umblliferae), which is cultivated chiefly in Minxian County, Gansu Province of China. Chinese people began to use Chinese Danggui for the treatment of gynaecological diseases over 2000 years ago, and the drug is now still widely used for many diseases.[1] Though some other herbs of the same genus, such as *Angelica acutiloba* (Siebold and Zucc.) Kitag, *Angelica gigas* Nakai, have also been used in medicine, *A. sinensis* is the most widely used and the best known among them.[2]

A previous study showed that the crude polysaccharide and a polysaccharide of low molecular weight from *A. sinensis* possessed anti-tumour effects, both in vitro and in vivo.[3,4] Moreover, the effects on blood coagulation, platelet aggregation and gastrointestinal protection of the crude polysaccharide were also reported.[5–7] So, the presence of such a compound could partly be responsible for the clinical effects of this plant.

Therefore, much attention has been paid to the extraction method and the structure elucidation of the polysaccharide from *A. sinensis*. Wang et al.[8] reported that the polysaccharide from *A. sinensis* was composed of fucose, galactose, glucose, arabinose, rhamnose and xylose (mole ratio: 1.0:13.6:15.0:8.7:21.3:3.7). Furthermore, a neutral polysaccharide (ASP1) and two kinds of acidic polysaccharides (ASP2, ASP3) were isolated from *A. sinensis* recently.[9] The composition analysis displayed that ASP1 was rich in glucose, galactose and arabinose. However, no detailed structural studies were carried out with any of the polysaccharides isolated from *A. sinensis*. In the present study, we successfully characterize two water-soluble glucans isolated from *A. sinensis* for the first time.

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2. Results and discussion

The crude polysaccharide, APS-0, was obtained by precipitation with ethanol and dialysis from the water extract of *A. sinensis*. APS-0 was further fractionated into an unabsorbed and two absorbed fractions on the DEAE-Sephadex A-25 column. The unabsorbed fraction (APS-1) was regarded as a neutral main polysaccharide as it was eluted with water, and no acidic sugar was found in this fraction. The absorbed fractions (APS-2 and APS-3) eluted with NaCl solution from anion-exchange chromatography were regarded as acidic polysaccharides. This is consistent with the results from the sugar composition analysis of APS-2 and APS-3, as galacturonic acid was found in considerable amounts (unpublished results). APS-1 was further fractioned by gel filtration on a Sephacryl S-400 column, and the major fraction, APS-1c, was purified by gel filtration on a Sephadex G-100 column to get two major fractions, APS-1cI and APS-1cII.

The major polysaccharides of the neutral fraction, APS-1cI and APS-1cII, showed symmetrical peaks on high-performance size-exclusion chromatography (HPSEC). The yields, protein content, sugar content, optical rotations and molecular weights of APS-1cI and APS-1cII are listed in Table 1. The weight-average molecular weights of APS-1cI and APS-1cII, determined from a calibration curve prepared with standard dextrans, were found to be $\sim 1.7 \times 10^5$ Da and $\sim 3.9 \times 10^4$ Da. The component sugar of APS-1cI and APS-1cII was determined to be only glucose.

The FTIR spectra of APS-1cI and APS-1cII are shown in Figure 1. The bands in the region of 3422.18 cm^{-1} are due to the hydroxyl stretching vibration of the polysaccharides. The bands in the region of 2929.79 cm^{-1} are due to C–H stretching vibration, and the bands in the region of 1642.27 cm^{-1} are due to associated water. Absorptions at 916.09 cm^{-1} are typical for D-Glc in the pyranose form.[10] Moreover, the positive specific rotations and the characteristic absorptions at 846.64 cm^{-1} in the IR spectra indicated α-configurations existing in both APS-1cI and APS-1cII.[11]

A modified methylation analysis of the APS-1cI gave only one peak of 1,5,6-tri-*O*-acetyl-2,3,4-tri-*O*-methyl-D-glucitol (*m*/*z*: 43, 58, 71, 87, 101, 117, 129, 161, 173, 189, 233).[12] The analysis of the methylated sugars was conducted by GC–MS of their alditol acetates.[13] Therefore, the results indicated that the α-glucan should be linear and only contain (1→6)-linked-D-glucopyranosyl.

The anomeric proton signal at δ 4.90 ($J_{H\text{-}1,H\text{-}2}$ 2.3 Hz) in the ^1H NMR (500 MHz) spectrum of APS-1cI confirmed that the sugar residues were α-glycosidically linked,[14,15] which was in good agreement with the presence of an IR band at 846.64 cm^{-1}. The proton signals from H-1 to H-6 were assigned using the $^1H/^1H$ homonuclear correlation (^1H–^1H COSY) experiment (Fig. 3). ^{13}C NMR (125 MHz) (Fig. 2) studies showed that all carbon lines were resolved. The carbon signals from C-1 to C-6 were identified from the $^1H/^{13}C$ heteronuclear single quantum correlation (HSQC) spectroscopy (Fig. 3) and the data available in the literature.[15–20] The anomeric carbon signal for the 1,6-D-Glc*p* residue was assigned at δ 97.7 ppm, which also confirmed that the sugar residue was α-glycosidically linked.[14] The C-6 signal at δ 65.5 ppm appeared at about 4 ppm downfield compared to that of the standard methyl glycoside

Table 1. Yields, protein contents, sugar contents, optical rotations and molecular weights (M_w) of APS-1cI and APS-1cII

Sample	Appearance	Yield[a]	Protein content (w/w)	Sugar content (w/w)	Optical rotation ($[\alpha]_D^{20}$ (*c* 0.2, H_2O))	M_w (Da)
APS-1cI	White, fluffy	19.2	4.0	93.5	+9.8	1.7×10^5
APS-1cII	White, fluffy	21.3	1.4	98.0	+175.4	3.9×10^4

[a] Calculated as weight % of APS-1c.

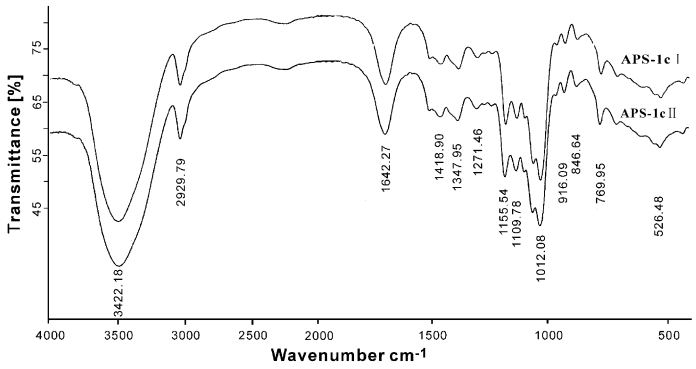

Figure 1. FTIR spectra of APS-1cI and APS-1cII.

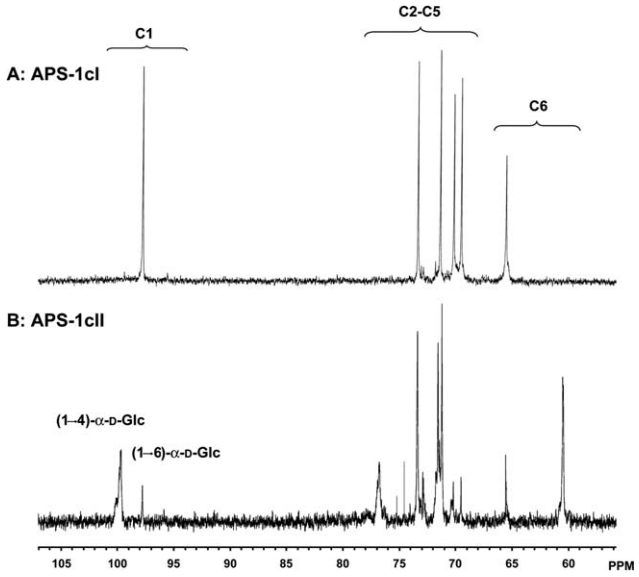

Figure 2. ^{13}C NMR spectra of APS-1cI and APS-1cII in D_2O at 30 °C: (A) APS-1cI; (B) APS-1cII.

value[21] due to the α-glycosylation effect. Signals for other carbons were: δ 73.4 (C-3), 71.4 (C-2), 70.2 (C-5) and 69.5 (C-4) ppm. All of these resonances agreed with a dextran.[16,20] Therefore, the following structure of APS-1cI was established:

$$[\rightarrow 6)\text{-}\alpha\text{-D-Glc}p\text{-}(1\rightarrow]_n$$

APS-1cII was also methylated, followed by hydrolysis and alditol acetate preparation and analyzed by GC–MS. The presence of 1,4,5-tri-*O*-acetyl-2,3,6-tri-*O*-methyl-D-glucitol (*m*/*z*: 43, 71, 87, 99, 101, 113, 117, 129, 161, 173, 233) and 1,5,6-tri-*O*-acetyl-2,3,4-tri-*O*-methyl-D-glucitol (*m*/*z*: 43, 58, 71, 87, 101, 117, 129, 161, 173, 189, 233) in a molar ratio of 4:1 was detected. This indicated that (1→4)-D-Glc and (1→6)-linked-D-Glc moieties were present in APS-1cII.

On periodate oxidation, the polysaccharide of APS-1cII showed abundance periodate uptake. The consumption of HIO_4 (1.14 mol) was about six times more than the amount of formic acid (0.18 mmol) per hexosyl residue, indicating the existence of monosaccharides that were (1→)-linked or (1→6)-linked. The periodate-oxidized products were hydrolyzed and examined by GC. There were two components, erythritol and glycerol with a molar ratio of 4:1, found in the GC spectrum. Since APS-1cII was only composed of glucose, the presence of erythritol indicated that a part of glucose was in either a (1→4)- or a (1→4,6)-linkage, while the presence of glycerol and formic acid suggested that a part of glucose was in a (1→6)- or a (1→)-linkage. In addition, no glucose was found by GC analysis, which indicated APS-1cII was not composed of (1→3)-Glc, (1→2,3)-Glc, (1→2,4)-Glc, (1→3,4)-Glc, (1→3,6)-Glc or (1→2,3,4)-Glc. These results were in agreement with the results of methylation analysis.

The ^1H NMR spectrum of APS-1cII showed two anomeric proton signals at δ 5.33 and 4.89 ppm. These were assigned as (1→4)-D-Glc*p* (residue A) and (1→6)-D-Glc*p* (residue B), respectively, in a molar ratio of 4:1, which was consistent with the results of the methylation analysis. In the ^{13}C spectrum of APS-1cII (Fig. 2), the anomeric carbon signals for the (1→4)-D-Glc*p* and (1→6)-D-Glc*p* residues were assigned at δ 99.9 and 97.8 ppm, respectively. The appearance of the respective carbon signals indicated that the (1→4)-D-Glc*p* and (1→6)-D-Glc*p* moieties were both at the α-anomeric configuration.[18] All the chemical shifts of APS-1cII in the ^{13}C NMR and ^1H NMR spectra are summarized in Table 2, which are assigned on the basis of the correlation of the HSQC (Fig. 4) and the total correlation spectroscopy (TOCSY) (Fig. 5) NMR experiments, and with reference to previous reports.[14–19,22] The carbon signal at δ 65.7 ppm should be C-6 of the (1→6)-D-Glc*p*, which was shifted about 4 ppm downfield compared with the resonance of standard methyl glycoside due to the effect of glycosylation.[20] Similarly, the C-4 signal at δ 76.9 ppm of (1→4)-D-Glc*p* appeared at 5.80 ppm downfield compared with that of the standard methyl glycoside.[21] The carbon signals at δ 73.4, 71.6, 71.3 and 60.7 ppm corresponded to C-3, C-2, C-5 and C-6 of (1→4)-α-D-Glc*p*, respectively. The other signals at δ 73.0, 71.8, 69.6 and 70.3 ppm corresponded to C-3, C-2, C-4 and C-5 of (1→6)-α-D-Glc*p*, respectively.

To deduce the sequence of glucopyranosyl residues of APS-1cII and to confirm the assignments made from the HSQC and TOCSY spectra, heteronuclear multiple-bond coherence (HMBC) (Fig. 6) and nuclear Overhauser effect spectroscopy (NOESY) (Fig. 5) experiments were used. In the HMBC spectrum, cross peaks of the H-1–C-4 (linkages of two residues A), the H-1–C-4 (linkages of residues B and residues A) and the H-1–C-6 (linkages of residue A and residue B) were identified. Moreover, there was no cross peak found between H-1 and C-6 of residue B. These results suggested that the (1→6)-D-Glc*p* should be linked directly to a (1→4)-D-Glc*p* residue. Moreover, the cross peaks from δ 5.33 to δ 3.43 ppm in HMBC spectrum also confirmed the assignments of signals within each glucosyl residue. Analogously, in the NOESY spectrum, the anomeric proton (H-1) of residue A had a strong interresidue NOE contact to H-6b and medium one to H-6a of residue B in addition to intraresidue NOE contacts to H-2, H-3, H-4, H-5 and H-6a. This indicated that residue A was linked at the 6-position of residue B. On the other hand, the H-1 of residue B had a strong interresidue NOE contact to H-4 of residue A in addition to intraresidue NOE contacts to H-2, H-3, H-4, H-5, H-6a and H-6b. So it was evident that residue B was linked at the 4-position of residue A. Hence, the linkages,

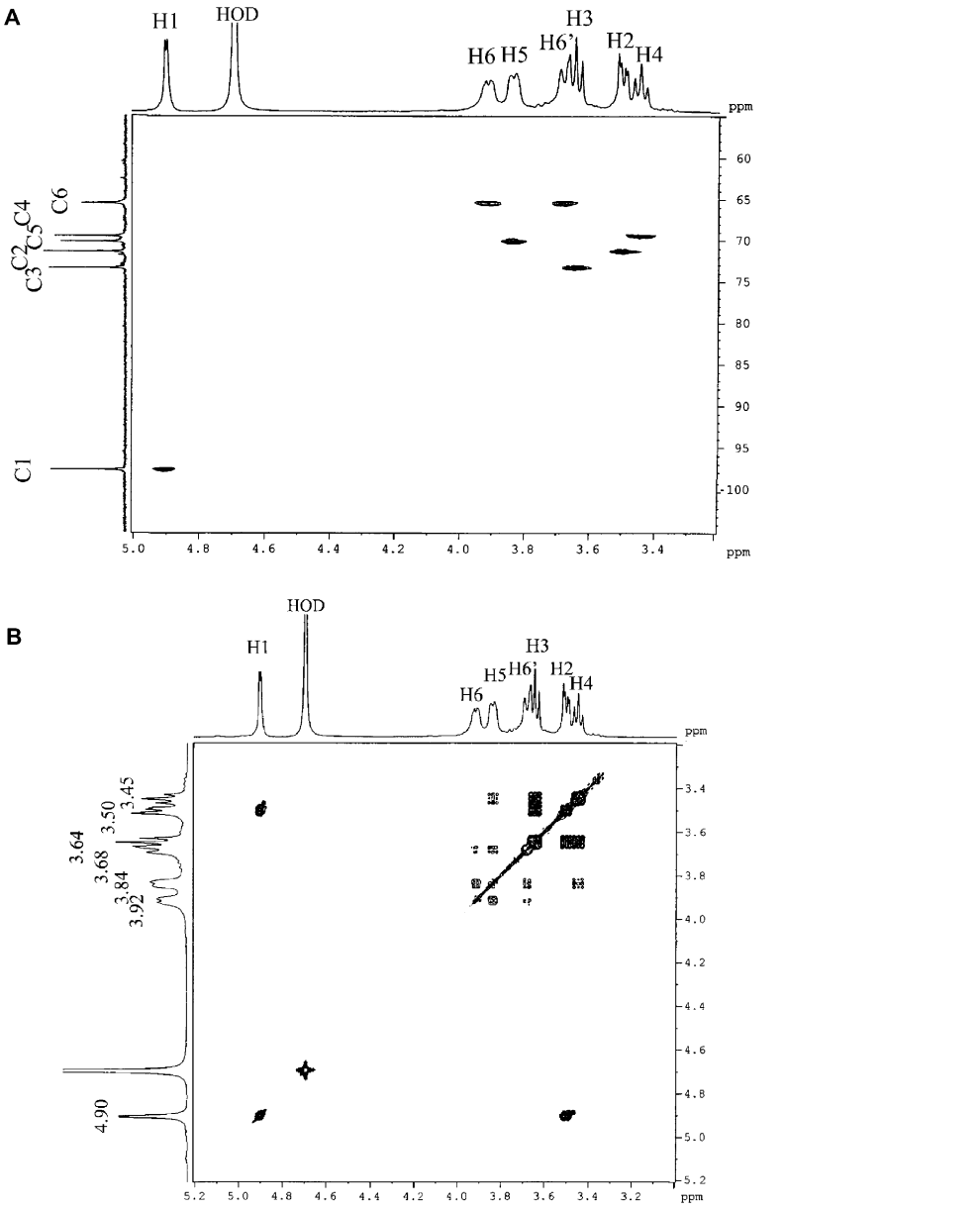

Figure 3. 500-MHz HSQC and H/H-COSY spectra of APS-1cI in D_2O at 30 °C: (A) HSQC; (B) H–H COSY.

Table 2. Summary of ^{1}H NMR and ^{13}C NMR chemical shifts for APS-1cII

Glycosidic linkage	Chemical shifts (ppm)						
	H-1/C-1	H-2/C-2	H-3/C-3	H-4/C-4	H-5/C-5	H-6/C-6	
→4)-α-Glc*p*-(1→	5.33/99.9	3.54/71.6	3.87/73.4	3.56/76.9	3.74/71.3	3.72[a] 3.70[b]	60.7
→6)-α-Glc*p*-(1→	4.89/97.8	3.48/71.8	3.63/73.0	3.43/69.6	3.84/70.3	3.92[a] 3.64[b]	65.7

[a,b] Interchangeable.

deduced from the HMBC spectrum were confirmed unambiguously as follows:

→4)-α-D-Glc*p*-(1→6)-α-D-Glc*p*-(1→

Since (1→4)-D-Glc*p* and (1→6)-D-Glc*p* were present in a molar ratio of 4:1, the following repeating unit for APS-1cII was established:

$$[\rightarrow 4)\text{-}\alpha\text{-D-Glc}p\text{-}(1\rightarrow 4)\text{-}\alpha\text{-D-Glc}p\text{-}(1\rightarrow 6)\text{-}\alpha\text{-D-Glc}p\text{-}(1\rightarrow 4)\text{-}\alpha\text{-D-Glc}p\text{-}(1\rightarrow 4)\text{-}\alpha\text{-D-Glc}p\text{-}(1\rightarrow]_n$$

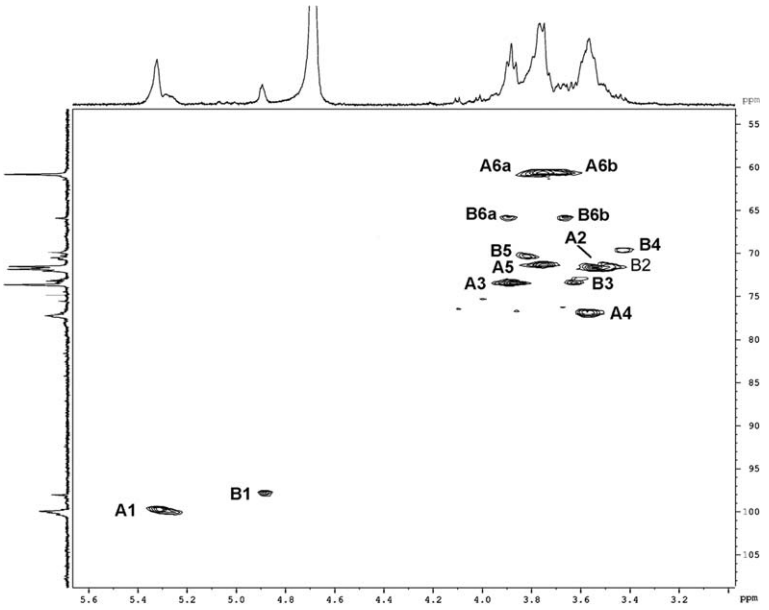

Figure 4. 500-MHz HSQC spectrum of APS-1cII in D_2O at 30 °C: (A) (1→4)-α-D-Glc*p*; (B) (1→6)-α-D-Glc*p*.

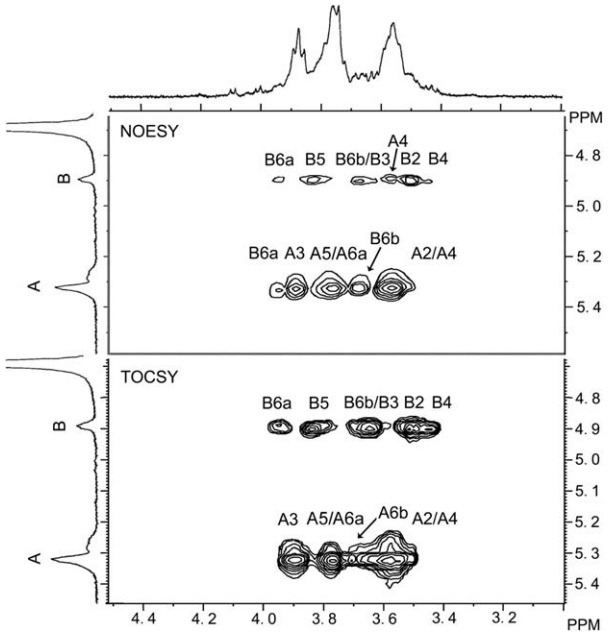

Figure 5. Key region of the TOCSY and NOESY spectra of APS-1cII. The mixing time for the TOCSY spectrum shown was 200 ms. The NOESY mixing delay was 80 ms.

In conclusion, this study described, for the first time, the structures of two water-soluble glucans isolated from the roots of *A. sinensis*. Although the polysaccharides composed of (1→6)-α-D-Glc*p* have been isolated from many kinds of living things, including plants, mushrooms, etc.,[23,24] APS-1cI is the first polysaccharide composed of (1→6)-α-D-Glc*p* isolated from Umbelliferae. The other glucan, APS-1cII was composed of a repeating unit consisting of (1→4)-α-D-Glc*p* and (1→6)-α-D-Glc*p* in a molar ratio of 4:1. This structure is similar to the structure of AG-1 isolated by Huang et al.[25] from *Aatragalus mongholicus*, except for the different molar ratio of (1→4)-α-D-Glc*p* and (1→6)-α-D-Glc*p*. It is also similar to a glucan characterized by Chakraborty et al.[14] from the fruit bodies of the mushroom *Astraeus hygrometricus*, except that the (1→6)-D-Glc*p* was of the β-configuration.

3. Experimental

3.1. Materials

The roots of *A. sinensis* were collected in Minxian County, Gansu Province, China, in October 2002 and identified by Professor X.F. Niu in the Department of Pharmacy, Xi'an Jiaotong University (Xi'an, China), by comparison with a voucher specimen deposited in the herbarium at the department. The coarse powder of the roots was air-dried in the shade and stored in a well-closed vessel for use.

T-series Dextran, DEAE-sephadex A-25, Sephacryl S-400 and Sephadex G-100 were purchased from Amersham Biosciences (Uppsala, Sweden). TFA was purchased from Sigma Chemical Co. (St. Louis, MO, USA). All other chemical reagents were of analytical reagent grade.

3.2. Extraction and fractionation of polysaccharide

The powdered roots (200 g) of *A. sinensis* were extracted with EtOH (400 mL) at 80 °C for 3 h × 3, in order to remove the pigments. The residue was extracted with boiling H_2O (800 mL) for 2 h × 3, filtered through gauze and

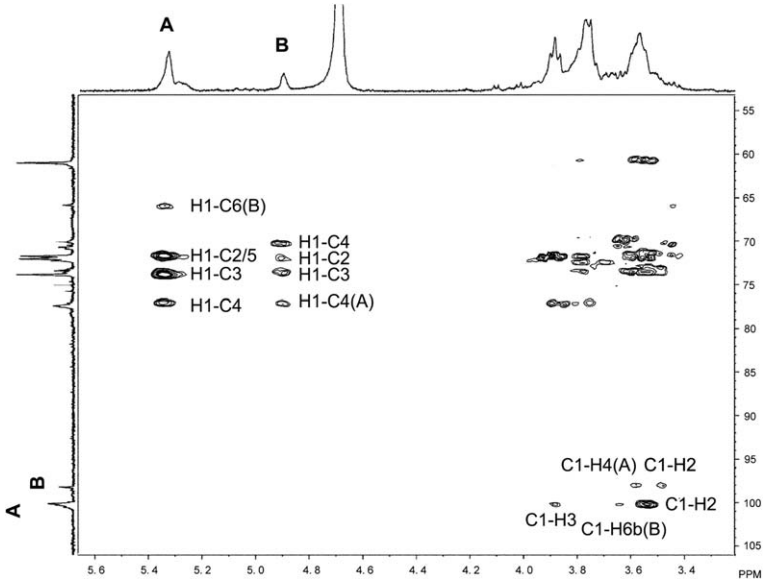

Figure 6. The HMBC spectrum of APS-1cII. Relevant cross peaks have been labelled.

centrifuged to remove water-insoluble materials. The aqueous extract was concentrated at 50 °C in vacuum and treated with 3 vols of EtOH for precipitation at 4 °C overnight. The gel-like precipitate was suspended in H_2O and dialyzed against distilled H_2O (exclusion limit 3.5 kDa). The nondialyzable portion was frozen at −20 °C, then thawed and centrifuged again to remove insoluble materials. After the freeze–thaw process was repeated six times, the supernatant was lyophilized, and a brown product was obtained (APS-0, yield: 4.4 g).

APS-0 was dissolved in distilled H_2O, filtered through a 0.65 μm membrane filter and loaded onto a DEAE-Sephadex A-25 column (80 cm × 3.5 cm). The column was first eluted with H_2O, followed by 0.3 and 0.5 M NaCl, respectively. Fractions of 10 mL were collected and monitored for the presence of carbohydrate using the phenol–H_2SO_4 assay.[26] Fractions containing carbohydrate were pooled, dialyzed and lyophilized. The neutral fraction from APS-0 designated as APS-1 (yield: 1.6 g) was further fractionated on a column (100 cm × 5 cm) of Sephacryl S-400, eluted with 0.1 M NaCl. The eluted solution was separated into three fractions (APS-1a, APS-1b and APS-1c) according to the elution profile. After being pooled, dialyzed and lyophilized, APS-1c (yield: 530 mg) was further fractionated on a column (100 cm × 3.5 cm) of Sephadex G-100, eluted with 0.1 M NaCl and separated into two purified polysaccharides (APS-1cI and APS-1cII) according to the elution profile. The relevant fractions were pooled, dialyzed and lyophilized.

3.3. General methods

Carbohydrate contents were measured by the phenol–H_2SO_4 method, using D-glucose as the standard.[26] Protein contents were measured by the Bradford[27] method using bovine serum albumin as standard. Optical rotations were measured at 20 °C using a Perkin–Elmer 343 polarimeter. Each sample was analyzed four times by the methods described above.

3.4. Monosaccharide analysis

APS-1cI and APS-1cII were hydrolyzed with 2 M TFA at 110 °C for 4 h.[28] Paper chromatography (PC) and gas chromatography (GC) were used for identification and quantification of the monosaccharides. PC was performed on Xinhua (Hangzhou, China) No. 1 paper using the EtOAc–*n*-BuOH–*i*-PrOH–HOAc–H_2O–pyridine (20:7:12:7:6:6) and visualized by spraying with phthalic acid reagent and heating at 100 °C for 15 min. The sugars in the hydrolysate were converted to their alditol acetates as described[29,30] and analyzed by GC on an Agilent 6890N instrument fitted with flame-ionization detector (FID) and equipped with an SE-54 column (30 m × 0.32 mm × 0.25 μm) at a temperature program as follows: 160 °C (10 min)–280 °C with a rate of 5 °C/min. The injector and detector heater temperatures were 250 and 300 °C, respectively. The rate of N_2 carrier gas was 1.2 mL/min.

3.5. NMR and FTIR spectroscopies

The polysaccharide (30 mg) was dissolved in D_2O (0.5 mL). The ^1H, ^{13}C, H–H COSY, HSQC, HMBC, TOCSY and NOESY NMR spectra were recorded using a Bruker 5-mm broadband observe probe at 30 °C with a Bruker Avance 500 MHz spectrometer (Germany). Chemical shifts are referred to the residual signal of HOD at δ 4.70 ppm for ^1H NMR spectra and the

external standard, Me_4Si for ^{13}C NMR spectra. All the experiments were recorded using standard Bruker software. Spectral width in the ^1H–^1H COSY spectrum was 1707 Hz in both dimensions. In the HSQC experiments, Echo/Antiecho gradient selection with decoupling was used. Spectral widths were 1707 and 11261 Hz for the proton and the carbon dimensions, respectively. The TOCSY experiment was recorded with a mixing time of 200 ms, and complete assignment required several TOCSY experiments requiring several mixing times ranging from 60 to 300 ms. The NOESY mixing delay was 80 ms. The HMBC experiment was recorded using gradient pulses for selection with a 71.4 ms delay for the evolution of the long-range couplings. FTIR spectra (KBr disc) were recorded with a Bruker Equinox 55 spectrophotometer for detecting functional groups.

3.6. Determination of homogeneity and molecular weights

The homogeneity and M_w of the polysaccharides were evaluated and determined by HPSEC using a Waters Alliance 2690 instrument equipped with a tandem arrangement of a Shodex sb-803HQ (8 mm × 30 cm) and a Biosep SEC-S3000 (Phenomenex, 7.8 mm × 30 cm) column, eluted with 0.05 M Na_2SO_4 at a flow rate of 0.8 mL/min. The elution was monitored by a Waters Alliance 2414 RI detector, and the data were analyzed with Millennium 32 (Waters Alliance) software. The columns were calibrated with standard T-series Dextran T-130, T-80, T-40, T-20 and T-10.[31]

3.7. Methylation analysis

Methylations of APS-1cI and APS-1cII were carried out three times using the method described earlier.[12] The methylated polysaccharides were examined by FTIR spectroscopy. Complete methylation was confirmed by the lack of a hydroxyl peak. The methylated products were hydrolyzed, reduced and acetylated as described by Sweet et al.[13] The resulting alditol acetates were subjected to GC and GC–MS analysis. Analyses were performed on an Agilent 6890N GC interfaced with an Agilent 5973N mass-selective detector at 70 eV ionization energy. The GC column was an HP-1 (30 m × 0.2 mm × 0.33 μm) at a temperature program from 140 to 280 °C with a rate of 3 °C/min. The rate of helium carrier gas was at a rate of 3.0 mL/min. The quantification for molar ratio for each sugar was calibrated using the peak area and response factor of the FID in GC.

3.8. Periodate oxidation

APS-1cII (20 mg) was added to 0.02 M $NaIO_4$ (50 mL), and the mixture was kept at 4 °C for 7 days in the dark.[32] Excess periodate was decomposed by addition of ethylene glycol (2 mL), and the consumption of $NaIO_4$ was measured by a spectrophotometric method.[33] The solution of periodate product (20 mL) was sampled to calculate the yield of HCO_2H by titration with 0.01 M NaOH. The rest was dialyzed against distilled H_2O for 24 h. The solution was concentrated and reduced with $NaBH_4$ (20 mg). The mixture was left for 24 h at room temperature, neutralized to pH 6.0 with HOAc, dialyzed, concentrated and hydrolyzed with 2 M TFA at 100 °C for 4 h. After the alditol acetate was prepared, the glycosyl residue composition of the resulting product from the oxidation of APS-1cII was analyzed by GC by the same methods as those used for the monosaccharide composition analysis.

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