Carbohydrate Polymers 87 (2012) 160–169

Contents lists available at ScienceDirect

Carbohydrate Polymers

journal homepage: www.elsevier.com/locate/carbpol

Structural characterization of a sulfated glucan isolated from the aqueous extract of *Hedysarum polybotrys* Hand.-Mazz

Yikai Shi [a], Lianggong Zhao [b], Xiaohua Liu [a], Fangdi Hu [a], Fang Cui [a], Yingyan Bi [a], Yufeng Ma [a], Shilan Feng [a,c,*]

[a] School of Pharmacy, Lanzhou University, 199 Donggangxi Road, Lanzhou 730000, PR China
[b] The Second Clinical Medical School of Lanzhou University, 82 Cuiying Men, Lanzhou 730030, PR China
[c] Key Lab of Preclinical Study for New Drugs of Gansu Province, Lanzhou University, 199 Donggangxi Road, Lanzhou 730000, PR China

ARTICLE INFO

Article history:
Received 26 February 2011
Received in revised form 10 July 2011
Accepted 20 July 2011
Available online 27 July 2011

Keywords:
Hedysarum polybotrys Hand.-Mazz
Sulfated glucan
Structure
NMR spectroscopy

ABSTRACT

A water-soluble sulfated glucan, SHG, was isolated from *Hedysarum polybotrys* Hand.-Mazz using anion-exchange and gel-permeation chromatography. Elemental analysis indicated that SHG was a sulfated polysaccharide containing small amount of sulfate groups (1.47 mass%). The molecular weight was estimated to be 1.72×10^5 Da by high-performance gel permeation chromatography (HPGPC) and size exclusion chromatography–multi angle laser light scattering (SEC–MALLS). SHG took random coil compact conformation in 0.1 M $NaNO_3$. Compositional analysis revealed that SHG was composed of glucose only. On the base of partial acid hydrolysis, methylation analysis, gas chromatography–mass spectrometry (GC–MS), Fourier transform infrared (FT-IR) spectra, nuclear magnetic resonance (NMR) spectroscopy of ^1H, ^{13}C, β-correlation spectroscopy (β-COSY), total correlation spectroscopy (TOCSY), heteronuclear single quantum coherence (HSQC), and heteronuclear multiple bond correlation (HMBC), the results showed that SHG was composed of α-D-(1 → 4)-linked glucopyranosyl residues, with branches at C-6 consisting of non-reducing terminal approximately every eight residues. Sulfate groups may attach to the backbone at O-6, occasionally occurring per thirty-eight anhydrous glucose units.

1. Introduction

Plant originated macromolecules have been intensively investigated for their biological activities. Among those, polysaccharides have gained as biological macromolecules and polysaccharides with a sulfate group play a predominant role. Interestingly, some sulfated polysaccharide derivatives exhibit antiviral action (Hatanaka et al., 1987; Tanaka et al., 1989). These sulfated polysaccharides contain hemi-ester sulfated groups in their sugar residues. Moreover, these compounds commonly found in marine algae and higher animals but are absent in microbes and higher plants (Shanmugam & Mody, 2000).

The dried root of *Hedysarum polybotrys* Hand.-Mazz, known as "Hongqi" in China, is a famous traditional Chinese medicine. It has a long history of use in the treatment of various diseases in China. It has been reported that the crude polysaccharides from *H. polybotrys* exhibit anti-cancer, anti-aging, anti-oxidation, anti-tumor, hypoglycemic, and immunological activities (Cui et al., 1998; Hu, Li, Zhao, Feng, & Wang, 2010; Huang et al., 1992; Jin et al., 2004; Jin & Zhang, 2006; Lan, Zhang, Cheng, Wang, & Xi, 1987; Li et al., 2008; Song et al., 2000; Wang, Ito, & Shimura, 1989; Zhou, Jin, Zhang, & Li, 2006).

In the present study, a water-soluble sulfated polysaccharide (designated SHG below) from *H. polybotrys* was isolated, purified, and characterized by a combination of chemical and instrumental analysis, such as sugar analysis, methylation analysis, 1D and 2D NMR spectroscopy including β-correlation spectroscopy (β-COSY), total correlation spectroscopy (TOCSY), heteronuclear single quantum coherence (HSQC), and heteronuclear multiple bond correlation (HMBC) experiments.

2. Materials and methods

2.1. Materials

The roots of *H. polybotrys* were purchased from Wudu, Gansu Province and identified by Prof. Zhigang Ma, School of Pharmacy, Lanzhou University. The voucher specimen was deposited in the herbarium of School of Pharmacy, Lanzhou University. The radix of *H. polybotrys* was air-dried in shade and ground into powder with a mill (60-mesh). DEAE-52 Cellulose was purchased from Whatman

* Corresponding author at: School of Pharmacy, Lanzhou University, 199 Donggangxi Road, Lanzhou 730000, PR China. Tel.: +86 931 8915686; fax: +86 931 8915686.
E-mail address: fengshl@lzu.edu.cn (S. Feng).

0144-8617/$ – see front matter © 2011 Elsevier Ltd. All rights reserved.
doi:10.1016/j.carbpol.2011.07.032

Co., U.K. The standard monosaccharides, including glucose, rhamnose, xylose, galactose, mannose, arabinose, glucuronic acid, were all from National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Dextrans of different molecular weights (668,000, 410,000, 273,000, 148,000, 48,600, 23,800, 11,600, and 5200 Da) were purchased from PSS (USA). Sephadex G-200, G-100 were purchased from Treechem (Shanghai, China). Unless otherwise stated, all of other reagents were all analytical grade.

2.2. General methods

Evaporations were performed on Eyela (Tokyo, Japan) under reduced pressure at a temperature less than 50 °C. The optical rotation was measured on a Perkin-Elmer 341 polarimeter (PerkinElmer, USA) in water at 25 °C. The solution was lyophilized using a lyophilizer (Labconco FreeZone, USA). Total carbohydrate content was analyzed by phenol-sulfuric acid method (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956), with glucose as the standard. Protein content was analyzed by Bradford method with bovine serum albumin as standard (Bradford, 1976). Uronic acid content was determined by the *m*-hydroxydiphenyl method (Blumenkrantz & Asboe-Hansen, 1973) using glucuronic acid as standard. The sulfate content was measured by elemental analysis. Dialysis was carried out using a 3500 Da molecular weight cut off membrane tubing (for globular protein). GC analysis was taken on a Shimadzu GC-2410 (Shimadzu, Japan) with flame ionization detector (FID), using a fused-silica capillary column OV-101 (50 m × 0.25 mm i.d × 0.33 μm film thickness).

2.3. Extraction and isolation

The dried roots of *H. polybotrys* were extracted by the following method with modifications, as previously described (Ma et al., 2008). Briefly, the ground roots (1000 g) were under reflux with 95% (v/v) ethanol to remove lipid. After removing the supernatant through gauze, the residue was air-dried, and then extracted two times with 10 vol of distilled water at 60 °C for 2 h each time. The pooled filtrate was concentrated to a proper volume under reduced pressure and centrifuged. The supernatant was precipitated by stepwise addition of chilled 95% (v/v) ethanol to final ethanol concentrations of 30%, 50%, 70% (v/v) and storage overnight at room temperature. The precipitates were accordingly collected by centrifugation (5000 rpm for 10 min, at 20 °C) and lyophilized to get three crude polysaccharide fractions, named as HPS1, HPS2, and HPS3. Savag method was used for the deproteination of HPS2 (Li, Hui, Feng, & OuYang, 2010). Then, the deproteined HPS2 was dialyzed and freeze-dried.

2.4. Purification

HPS2 (180 mg) was dissolved in distilled water, centrifuged, and then the supernatant was applied to a DEAE-52 cellulose column (2.6 cm × 30 cm) equilibrated with distilled water. The column was eluted stepwise with distilled water, 0.1 M, and 0.3 M NaCl aqueous solution, and fractions collected at a flow rate of 0.4 mL/min. Then, 4-mL fractions were collected. The collected fractions were pooled, dialyzed and freeze-dried.

The partially purified polysaccharide (100 mg) was performed on a Sephadex G-200 column (3.4 cm × 50 cm), equilibrated with distilled water. The column was eluted with distilled water at the flow rate of 0.5 mL/min. 5-mL fractions were collected. Test tubes (100 containing 8 mL eluent each) were collected using an automated step-by-step fraction collector. The fractions were pooled, dialyzed and freeze-dried to obtain purified polysaccharides for the subsequent analysis. The purification process was carried out in several lots as described above.

2.5. Elemental analysis

Elemental analysis was carried out using an Elementar Vario EL instrument (Elementar, Germany) to analyze carbon (C), hydrogen (H), oxygen (O), sulfur (S) and nitrogen (N). Sulfate content ($-SO_2O^-Na^+$, sodium salt) was calculated from sulfur analysis according to the following relation: sulfate group = 3.22 × S%.

2.6. Homogeneity and molecular weight

2.6.1. HPGPC

The homogeneity and molecular weight of polysaccharides were estimated using HPGPC method, which was performed on a Waters HPLC module consisting of a Model 600 pump, an RI detector (Model 2414) and a photodiode array detector (PAD, Model 2998). The column was a serially linked combination of an Ultrahydrogel™ 1000 and an Ultrahydrogel™ 500 column. Deionized water was used as the eluent for samples, with the flow rate kept at 0.8 mL/min. The columns and RI detector temperature were maintained at 40 and 35 °C, respectively. The columns were calibrated by reference to the MW-known T-series dextrans (668,000, 410,000, 273,000, 148,000, 48,600, 23,800, 11,600, and 5200 Da). Data were processed by GPC software (Empower). A sample volume of 20 μL (0.5 mg/mL) was injected for each run.

2.6.2. SEC–MALLS

Homogeneity and molecular weight were also determined by SEC–MALLS method. SEC–MALLS measurements were carried out on a DAWN HELEOS-II laser photometer (DAWN HELEOS-II, Wyatt Technology Co., Santa Barbara, CA, USA), combined with two Shodex OH pak SB-804 and 803 columns (8.0 mm × 300 mm, Showa Denko k.k., Japan) and equipped with a series 1500 pump HPLC system (SSI, USA), differential refractive index detector (DRI, Optilab rEX, Wyatt) and UV detector (SSI 500). The calibration of the laser photometer was done with ultra pure toluene and the normalization was done with polystyrene (PS, $M_w = 2.0 \times 10^4$ Da, $M_w/M_n = 1.10$, APSC, USA) at the concentration of 0.15–0.75 mg/mL. The calibration of the interferometric refractometer was made with NaCl aqueous solution. The average value of refractive index increment (dn/dc) was determined to be 0.135 mL/g using the interferometric refractometer at 633 nm. Astra software (Version 5.3.4) was utilized for data acquisition and analysis. Columns temperature was maintained at 40 °C, and DRI detector temperature was maintained at 35 °C.

SHG was dissolved in 0.1 M aqueous $NaNO_3$ and set aside overnight. Solutions (200 μL, 3 mg/mL) were injected and eluted at 0.8 mL/min with 0.1 M $NaNO_3$, containing 0.02% NaN_3, as a mobile phase. The solution and mobile phase were dust-free treated by passing through 0.2 μm pore size filter membrane and degassed before being used.

2.7. Sugar analysis

2.7.1. TLC

SHG (15 mg) was hydrolyzed with 2 M trifluoroacetic acid (TFA, 2 mL) in a sealed tube at 100 °C for 10 h. The excess acid was completely removed under reduced pressure by repeated co-evaporations with methanol. Then, the hydrolyzed product was divided into two parts for following analysis. One part was examined by thin-layer chromatography (TLC). Another part was analyzed by gas chromatography (GC) as aldononitrile acetates.

TLC was performed on thin layer plate (5‰ CMCNa silica gel G, containing 0.3 mol/L NaH_2PO_4, 5 cm × 20 cm) with a solvent system

of ethyl acetate, glacial acetic acid, methanol and H_2O in a ratio of 8:2:2:1 (v/v/v/v). The developing procedure repeated once. Rhamnose, xylose, arabinose, mannose, glucose, and galactose (5 mg/mL in water) were used as standard sugars. Sugar spots were visualized by spraying aniline–diphenylamine reagent with heating at 80 °C for 10 min.

2.7.2. Neutral sugar composition as aldononitrile acetates

The hydrolysates were converted to acetylated aldononitrile derivatives according to conventional protocols. The above dried product was acetylated with 0.5 mL pyridine adding 10 mg hydroxylammonium and 7 mg myo-inositol at 90 °C for 30 min, in parallel with standards. Acetic anhydride (0.5 mL) was then added to the mixture and the solution was incubated at 90 °C for another 30 min. Finally, the per-*O*-acetylated aldononitrile was partitioned between methylene chloride (2 mL) and water (2 mL). The methylene chloride layer was transferred to a clean tube and the methylene chloride was evaporated to give aldononitrile acetates, then 1 mL chloroform was added. Gas chromatography was performed on a GC-2410 instrument with myo-inositol as the internal standard. They were identified according to their retention times and quantified using an internal standard method involving myo-inositol.

The GC operation was performed using the following conditions: injector temperature: 210 °C; injection mode: split; split ratio: 20.0; injection volume: 2 μL; carrier gas: nitrogen; detector temperature: 240 °C. Temperature program: 110 °C, held for 5.0 min, to 190 °C at 5.0 °C/min, held for 4.0 min, then to a final temperature of 210 °C at 3.0 °C/min, held for 20.0 min.

2.7.3. Sugar composition as trimethylsilyl derivatives

Methanolysis and trimethylsilylation were performed as previously described (Doco, O'Neill, & Pellerin, 2001; York, Darvill, McNeil, Stevenson, & Albersheim, 1986).

SHG (0.1 mg) and monosaccharides were dried in a desiccator over P_2O_5 and transferred to sealed tubes. Then 20 μg of *n*-eicosane was added as an internal standard. Methanolysis was performed by adding 2.4 mL of 1 M methanolic HCl at 80 °C for 20 h. Thereafter, the cooled solvent was removed using a nitrogen stream at 40 °C. The resulting methyl glycosides were converted to the corresponding trimethylsilyl derivatives by York et al. (1986). Subsequently, the reactives were removed and the residue was immediately dissolved in 0.5 mL of hexane. GC analysis was performed with 2 μL of this solution prior to centrifugation. Separation and quantification of the per-*O*-trimethylsilyl methyl glycosides was performed on a GC-2410 system by comparison with the corresponding standards.

The GC operation was performed using the following conditions: the temperature program was used: 180 °C at 2.0 °C/min and finally 250 °C holding for 10 min. Injector temperature: 230 °C; injection mode: split; split ratio: 20.0; injection volume: 2 μL; carrier gas: nitrogen; detector temperature: 250 °C.

2.8. Partial acid hydrolysis

SHG (100 mg) was hydrolyzed with 0.1 M TFA (50 mL), kept at 100 °C for 1 h. After TFA was removed by evaporation, the remains were dialyzed with distilled water for 48 h. The solution was centrifuged, and the supernatant in the sack was freeze-dried, and then applied on Sephadex G-100 column (2.6 cm × 70 cm). The column was eluted with distilled water at the flow rate of 0.4 mL/min and fractions (4 mL) were collected. This pool was concentrated, and lyophilized to obtain sub-fraction. The sub-fraction in the sack (the backbone of SHG) was designated as SHGp. To establish the sequence of linkages in SHG, SHG and SHGp were subjected to the conventional methylation method and NMR analysis as described below.

2.9. Methylation analysis

Methylation analysis of SHG and SHGp was performed for four times using a modification of the Hakomori procedure (Hakomori, 1964).

SHG and SHGp (4 mg) were put in flasks (5 mL) and 3 mL 4 Å molecular sieve-dried dimethylsulfoxide (DMSO) were added, respectively. Then, the mixture was stirred at room temperature overnight, to which sodium dimethylsulfinyl anion (SMSM, 1.5 mL) solution was added and the mixture was then treated by sonication attached to an ultrasonic cleaner (KQ-3200DE, Kunshan, China, 40 kHz) for 30 min (20–25 °C). After incubation overnight at room temperature, methyl iodide (1.5 mL) was added to the mixture under ice bath and sonicated for a further 1 h at room temperature. The methyl iodide was removed by evaporation under a stream of nitrogen. The reaction was terminated with the addition of water. The reaction solution was extensively dialyzed against distilled water, and freeze-dried to obtain methylated polysaccharides. The methylation procedure was under the protection of N_2.

The completion of methylation was confirmed by IR spectroscopy as the disappearance of OH bands.

The complete methylated polysaccharides were treated with 88% formic acid (2 mL) for 6 h at 100 °C in a sealed tube. After removal of formic acid by rotary evaporation, residues were heated with 2 M TFA (3 mL) for 6 h at 100 °C and the resulting hydrolysates rotary evaporated to dryness. Thereafter, the hydrolysates were dissolved in 2 mL of distilled water to reduce by adding 25 mg of $NaBH_4$ at room temperature for 2 h. 100 μL of glacial acetic acid was used to terminate the reduction and then acetylated by addition of 2 mL of acetic anhydride at 100 °C for 1 h. The acetylated derivatives were evaporated and extracted with $CHCl_3$ (3 mL). The extract was washed with 3 mL water for three times and passed through a sodium sulfate column (0.5 cm × 10 cm) to remove water. The solvent was evaporated by a stream of nitrogen, and the residue was dissolved in 0.3 mL $CHCl_3$.

Partially methylated alditol acetates were identified by their fragment ions in EI-MS, and the molar ratios were estimated from the peak areas and response factors (Sweet, Shapiro, & Albersheim, 1975).

GC–MS analysis was performed on Shimadzu GC–MS Model QP-2010 Plus automatic system (Shimadzu, Japan) fitted with a fused silica Rxi-5 ms capillary column (30 m × 0.25 mm i.d × 0.25 μm film thickness). Injector temperature: 250 °C; injection mode: split; split ratio: 50.0; injection volume: 1 μL; carrier gas: helium, 1.0 mL/min; temperature program: 170 °C, held for 2.0 min, then to 190 °C at 2.0 °C/min, finally to 250 °C at 10.0 °C/min, held for 5.0 min. Ionization mode: electron impact at 70 eV; temperature of ion source: 200 °C; interface temperature: 250 °C; acquiring mode: scan, from *m*/*z* 43 to *m*/*z* 630; solvent delay: 4.0 min.

2.10. Spectroscopy

2.10.1. UV and FT-IR

SHG was dissolved with distilled water (1 mg/mL) and scanned on a Perkin Elmer Lambda 25 Uv–vis spectrometer from 190 nm to 800 nm.

SHG and SHGp were mixed with KBr pellets for FT-IR measurement and recorded on a Nicolet Nexus 670 spectrometer (Nicolet, USA) in the frequency range of 4000–400 cm^{-1}.

2.10.2. NMR spectroscopy

The freeze-dried SHGp (37.4 mg) was kept over P_2O_5 in desiccator for several days and lyophilized four times with D_2O (99.96%). Then the deuterium-exchanged polysaccharide was dissolved in 0.7 mL 99.96% D_2O, centrifuged, transferred in a 5-mm NMR tube for NMR analysis. The ^1H and ^{13}C NMR spectra were recorded

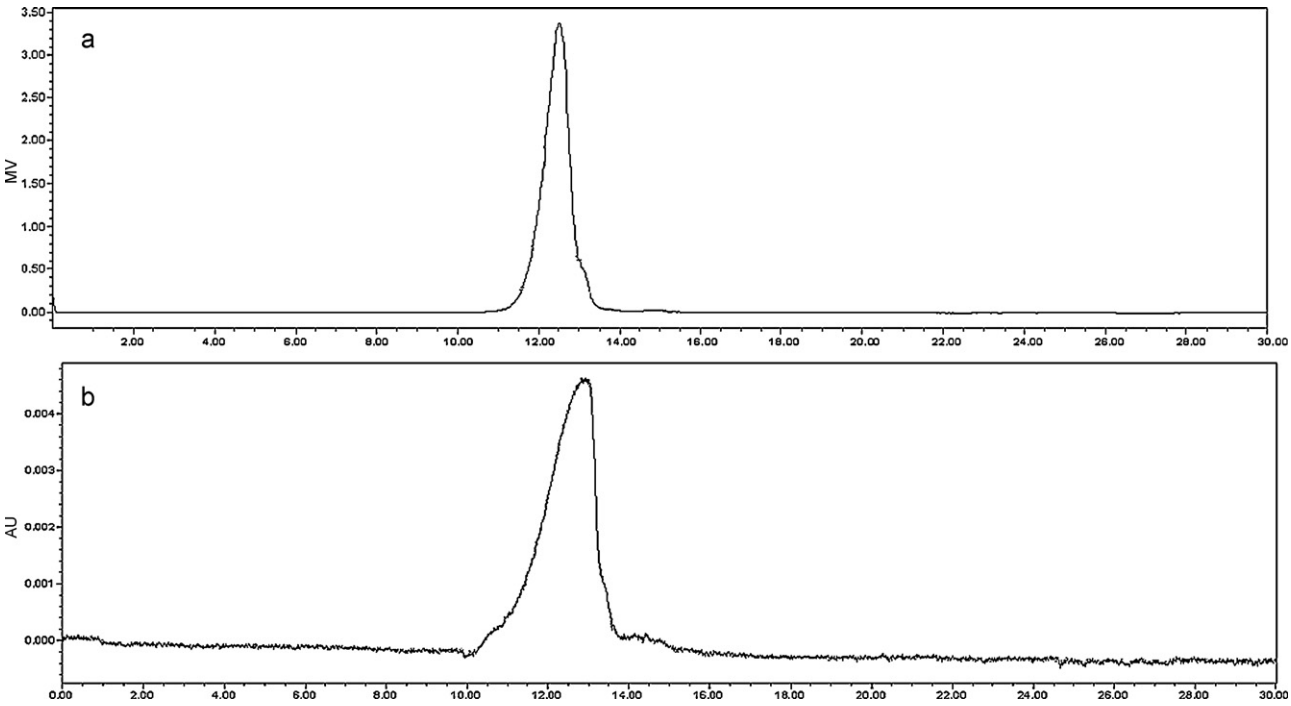

Fig. 1. HPGPC elution profile of SHG using a refractive index detector and photodiode array detector. (a) Profile of RI detector; (b) profile of photodiode array detector at 260 nm.

at 25 °C on a Bruker DRX-600 NMR spectrometer operating at 600 MHz for δ_H and 150 MHz for δ_C. The HOD peak was used as the internal reference at δ_H 4.62 for ^{1}H NMR spectroscopy. The chemical shifts were expressed in δ (ppm) values relative to TTS (tetramethylsilane) as external standard.

The β-COSY experiment was 1280 Hz in both dimensions. The TOCSY experiment was recorded at a mixing time of 80 ms. In HSQC experiment, spectral widths were 1280 and 7000 Hz for the proton and the carbon dimensions. The HMBC spectrum was recorded using a delay time of 80 ms. The 2D NMR experiments (β-COSY, TOCSY, HSQC, and HMBC) were performed using the standard Bruker Software.

3. Results and discussion

3.1. Isolation, purification of polysaccharides

The dried roots of *H. polybotrys* were defatted in 95% ethanol, and the residues were dried in air followed by extraction with hot water. Then the concentrated supernatant was precipitated with 95% ethanol (to give 30%, 50%, 70%, v/v final ethanol concentrations) to achieve three crude polysaccharides accordingly, denoted as HPS1, HPS2, and HPS3. The crude polysaccharides were lyophilized to obtain as yellow powder, with their yield being 1.48% (HPS1), 2.29% (HPS2), and 1.03% (HPS3), respectively. The polysaccharide contents of HPS1, HPS2, and HPS3 were found to be 20.23%, 22.30% and 45.37% respectively as determined by the phenol-sulfuric acid method.

HPS2 was further dissolved in distilled water and deproteined by Savag method. Then, the deproteined HPS2 centrifuged, exhaustively dialyzed, and freeze-dried. Upon anion-exchange chromatography on a DEAE-52 cellulose Cl^- column, three fractions (HPS2a, HPS2b, and HPS2c) were obtained from HPS2 by stepwise elution with distilled water, 0.1 M NaCl, and 0.3 M NaCl. Fractions corresponding to peaks were pooled and freeze-dried. One main fraction (HPS2b, eluted at higher ionic strength) was further purified on a Sephadex G-200 column and eluted with distilled water. The major fractions were then pooled and lyophilized to obtain dry polysaccharide, named as SHG. The purification process was carried out in several lots, yielded 401.34 mg.

SHG appeared as a white fluffy material. The total carbohydrate content of SHG was 97.19% measured by phenol-sulfuric acid method. The relatively high positive value of optical rotation of $[\alpha]_D^{25} + 164.19^\circ$ (c 0.2, H_2O) suggested the presence of α form glycosidic linkage, in agreement with the characteristic IR absorption band at 852.6 cm^{-1} which indicated the α-configuration of the glucosyl motif (Mathlouthi and Koenig, 1986).

I_2–KI reaction was negative, indicating no starch structure was detected. The negative result of *m*-hydroxydiphenyl reaction suggested that uronic acid was absent. SHG had a negative response in the Bradford test, indicating that the absence of protein. On a contrary, a broad absorption at 260–280 nm in the UV spectrum (Fig. 1 (b), Fig. 2 (a), UV spectrum at 260 nm), indicating the presence of protein or nucleic acid. This abnormal phenomenon really confused us. To confirm whether protein or nucleic acid was present in SHG, elemental analysis of SHG was done. The result of elemental analysis of SHG revealed that there were mainly C (40.11 mass%), H (6.65%), O (52.77%), S (0.47%), and N (0.00%), and the molar ratios of C/H/O were around 1:2:1, which demonstrated the absence of protein or nucleic acid. The absorption at 260–280 nm in UV was contributed to sulfate group, in agree with elemental analysis and FT-IR (described below). The total sulfate content ($-SO_2O^-$) was 1.47%. Approximately one sulfate ester was present per 38 sugar units on average.

3.2. Molecular mass and chain conformation

3.2.1. GPC

SHG showed a single and symmetrically sharp peak in HPGPC profile (Fig. 1), indicating it was a homogeneous polysaccharide. The calibration curve was linear, equation $\log M_w = s - 3.87e{-}001T^1 + 2.72e{-}003T^2$, with a correlation coefficient of $R^2 = 0.9972$. According to the retention time and the calibration curve with standard dextrans, it was calculated to have a molecular weight of over 6.6×10^5 Da.

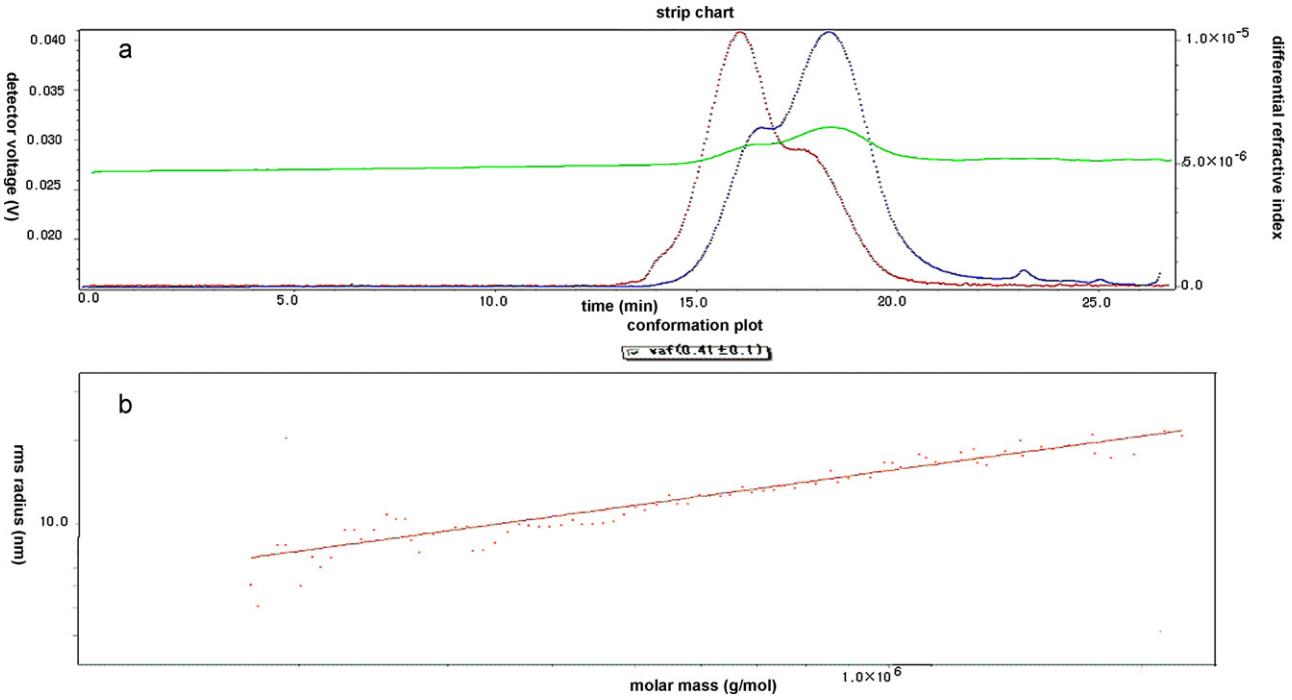

Fig. 2. (a) Profile of SEC–MALLS assay of SHG. Light scattering (LS) signal at 90° (red), UV signal at 260 nm (green), DRI signal (blue). (b) Conformation plot of rms radius vs M_w for SHG in 0.1 M aqueous $NaNO_3$ at 40 °C. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

3.2.2. SEC–MALLS

The characterization of natural polymers having various chemical components, molar mass, and chain conformation is important because of their critical effect on end-use structure–property relations (Cui et al., 2008). SEC–MALLS, as an absolute method, are convenient for the determination of true molecular mass and distribution without standard samples, which is extremely sensitive to macromolecular aggregation.

The SEC–MALLS chromatogram patterns of SHG in 0.1 M aqueous $NaNO_3$ at 30 °C were shown in Fig. 2(a), there was a single peak (with a shoulder) with good symmetry detected by LLS, UV at 260 nm and refractometer for SHG.

The molecular weight and chain conformation of SHG were determined with SEC–MALLS. The radius of gyration value, weight average molecular weight, and polydispersity index (M_w/M_n, M_n = 88.73 kD, M_w = 172.0 kD) in 0.1 M aqueous $NaNO_3$ were measured to be 9.0 nm (R_z), 1.72×10^5 Da, and 1.94, respectively. According to their estimated molecular weight, it was possible assume that SHG contained approximately 1048 sugar residues on average.

From data of M_w and the radius of gyration, the conformation plot describing the relationship between M_w and the radius of gyration can thus be created. Usually, the chain conformation of a polymer in solution can be judged by the conformation plot. The conformation plot is 0.3 for a globular shape, 0.5–0.6 for a flexible chain conformation, and 0.6–1 for a semi-flexible chain. The conformation plot for SHG was 0.45, indicating that the glucan existed as a compact random coil in 0.1 M $NaNO_3$ at 40 °C. The conformation plot of rms radius of gyration vs M_w for SHG in 0.1 M $NaNO_3$ was shown in Fig. 2(b).

There was a discrepancy in molecular weight values of SHG measured by SEC–MALLS in 0.1 M aqueous $NaNO_3$ solution (1.72×10^5 Da) and HPGPC in distilled water (over 6.6×10^5 Da). One explanation for the discrepancy in molecular mass on SHG could be that there was aggregation of SHG in distilled water but not in aqueous $NaNO_3$ solution. Polysaccharides often have high molecular weights, and tend to form aggregates in solution that can mask the behavior of individual macromolecules. Another explanation could be that SHG displayed an electrostatic repulsion effect in pure water, whereas they exhibited normal polymer solution behavior in 0.1 M aqueous $NaNO_3$ solution, proving that an electrostatic repulsion effect could be screened out successfully by adding salts. The electrostatic repulsion caused by macromolecule anions (sulfate group in SHG), leading to the more expanded chains. Therefore, as $NaNO_3$ was added, the electrostatic repulsion could be screened out. For macromolecules of the same molecular mass, a more compact random coil chain conformation gives a lower extent of expansion of the chain in solution. HPGPC is a relative method according molecular size but not molecular weight, the molecular configuration and size are changed along with electrolyte concentration in mobile phase. Therefore, SHG has such different molecular weight (Wang & Zhang, 2009; Zhang, Yang, & Fang, 1997). In short, the disagreement between SEC–MALLS and HPGPC in molecular weight was contributed to sulfate group.

Although the results are controversial and strongly depended on the method used for analysis, the authors believe that the result of SEC–MALLS as an absolute method is much more probable based on explanations as foresaid. Thus the molecular mass and chain conformation can be further studied.

3.3. Sugar analysis

Compositional analysis of SHG was determined by TLC preliminarily. Through comparing with standards, the monosaccharide composition was identified, a single spot on thin layer plate was observed. The investigation indicated that SHG was composed of only glucose, and no uronic acid was detected.

Monosaccharide composition of SHG also was determined by GC of aldononitrile acetates. The result showed that SHG was composed of glucose only, comparing the retention time with standards.

The aldononitrile acetates GC method cannot detect uronic acids unless the carboxyl groups of glucouronic acids have been reduced. Uronic acids can be quantified either by a colorimetric determination or by gas chromatography after methanolysis and subsequent trimethylsilylation. Neutral sugars and uronic acids can be

Table 1
Methylation analysis data for SHG and SHGp.

Methylated sugar	Molar ratios		Diagnostic mass fragments (m/z)	Type of linkage
	SHG	SHGp		
2,3,6-Me_3-Glc	7.2	7.4	43, 45, 87, 99, 117, 129, 162, 173, 203, 233, 277	→4)-Glcp-(1→
2,3,4,6-Me_4-Glc	1	1	43, 45, 101, 117, 129, 145, 161, 205, 249	Glcp-(1→
2,3-Me_2-Glc	1	1	43, 85, 87, 101, 117, 127, 142, 159, 161, 201, 261	→4,6)-Glcp-(1→

2,3,4,6-Me_4-Glc = 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol, etc.

simultaneously determined using GC of TMSi derivatives. Result from GC of trimethylsilylated methyl glycosides, showed that SHG contained Glc without any uronic acid material.

Base on these results, compositional analysis of SHG, determined by TLC and GC analysis, revealed that glucose was the only component in SHG, and SHG did not contain any uronic acid material.

3.4. Partial acid hydrolysis

The analysis of polysaccharides in their native form is difficult due to their high molecular weight. In order to overcome this, polysaccharides are usually cleaved into oligosaccharides or polymers with lower degree of polymerization. Partial acid hydrolysis approach has been used in order to obtain polymers with lower molecular weight suitable for structural characterization.

SHG was partially hydrolyzed with 0.1 M TFA. The hydrolyzate was centrifuged, and then the supernatant was exhaustively dialyzed against distilled water. After elution of Sephadex G-100, the fractions were collected, named as SHGp. Based on SEC–MALLS, its molecular weight, polydispersity (M_w/M_n) was 1.93×10^4 Da, 1.89. SHGp (yielded of 72.2 mg, 66.79%) was subsequently subjected to methylation and NMR analysis due to its lower molecular weight.

3.5. Methylation analysis

Methylation of SHG and SHGp were performed four times to obtain fully methylated polysaccharides using the modified Hakomori method. The OH absorption at 3400 cm^{-1} in IR disappeared, indicating the completeness of methylation. After hydrolysis and alditol acetate derivatization, the partially methylated alditol acetates were analyzed by GC–MS. Peaks of methylated sugars were identified by their mass spectra. The relative molar ratios of methylated fragments were calculated based on the peak areas of the methylated products and corrected using the effective-carbon response method (Sweet et al., 1975). Methylation analysis proposed the presence of three components. The methylation analysis results (Table 1) indicated that the presence of 2,3,6-tri-*O*-methyl-D-glucitol (m/z 43, 45, 87, 99, 117, 129, 162, 173, 203, 233, 277), 2,3-di-*O*-methyl-D-glucitol (m/z 43, 85, 87, 101, 117, 127, 142, 159, 161, 201, 261), 2,3,4,6-tetra-*O*-methyl-D-glucitol (m/z 43, 45, 101, 117, 129, 145, 161, 205, 249) in a molar ratio of nearly 7:1:1. SHG and SHGp had the similar results, so it indicated that the repeating unit of SHG was unchanged during partial acid hydrolysis. These results indicated that this glucan contained a backbone chain composed essentially of (1 → 4)-linkages with side branching on C-6 deduced from the existence of 1,4,6-Glc linkages.

3.6. FT-IR

FT-IR spectroscopic analysis (Fig. 3) of SHG and SHGp showed a strong band at 3422.76 cm^{-1} attributed to O–H stretching vibration. The band at 2926.42 cm^{-1} was due to C–H stretching vibration. The broad band at 1638.97 cm^{-1} was due to bound water (Park, 1971). In addition, no absorption peaks at 1730 cm^{-1} indicated that there were no uronic acids.

The bands at 1241.80 cm^{-1}, 1369.98 cm^{-1}, 580 cm^{-1} were assigned respectively to asymmetric and symmetric stretching vibrations of O–S–O that was an evidence of sulfate esters (Mahner, Lechner, & Nordmeier, 2001; Melo et al., 2002). The feeble band at 1241.80 cm^{-1} describing an asymmetrical S=O stretching vibration indicated low content of sulfate. The region around 800–850 cm^{-1} was used to infer the position of the sulfate group (Chopin & Whalen, 1993; Lahaye & Yaphe, 1988). However, besides the C–O–S vibration in 820–850 cm^{-1} region, there was also C–H bending vibration of sugar reducing end, and the content of sulfate was too small, which affected the judgment on the position of sulfate group in SHG (Li, Gu, & Xu, 2004).

The band at 852.65 cm^{-1} was ascribed to α-type glycosidic linkages, absorptions at 931.16 and 762.82 cm^{-1} were typical signal for D-Glc in the pyranose form (Barker, Bourne, Stacey, & Whiffen, 1954). Therefore, the FT-IR spectroscopy results, together with the high positive specific rotation, indicated the presence of α-D-glycosidic linkages in SHG. The absorptions at 1025.23, 1082.57, and 1154.86 cm^{-1} also indicated a pyranose form of the glucosyl residue. These results agreed with α-D-(1 → 4)-linked glucose residues in SHG.

3.7. NMR spectroscopy

The presence in the spectrum of well defined and sharp peaks testified the high purity of the sample. In the low field region, no typical signals were observed for the carboxyl group of the uronic acid units. The spectra were interpreted according to the literature values (Delben, Forabosco, Guerrini, Liut, & Torri, 2006; Ghosh, Chandra, Ojha, & Islam, 2008; Roslund, Tahtinen, Niemitz, & Sjoholm, 2008; Seymour, Knapp, Chen, Jeans, & Bishop, 1979; Yamada et al., 1986).

The 600 MHz ^{1}H NMR spectrum of SHGp (Fig. 4(a)) showed three anomeric proton signals at δ 5.26, 5.21 (unresolved doublet, 5.22, 5.20 ppm), and 4.83 ppm, which were designated as A (→4)-α-D-Glcp-(1→), B (→4,6)-α-D-Glcp-(1→), and C (α-D-Glcp-(1→), respectively, according to their decreasing anomeric chemical shifts. Consistent with presence of an IR band 852.65 cm^{-1}, this confirmed that the sugar residues were α-glycosidically linked glucan (Chakraborty, Mondal, Pramanik, Rout, & Islam, 2004). According to the peak areas, integration data indicated that three types of residues were in the ratio of nearly 7:1:1. This was also in accordance with the results of methylation analysis. The ^{13}C NMR (150 MHz) spectrum of SHGp showed that three anomeric carbon signals appeared at δ 102.14 (A), δ 101.11 (B), and δ 98.29 (C) ppm in a molar ratio of approximately 7:1:1. The α-configuration of the D-glucosyl groups was again conformed by the presence of three anomeric peaks, in the regions of δ 102.14, 101.11 and 98.29 ppm from ^{13}C NMR spectrum of SHGp (Fig. 4(b)).

The anomeric proton signal of residue A at δ 5.26 ppm indicated that it was an α-linked residue. The downfield shift of C-4 (79.27 ppm) with respect to standard value (Agarwal, 1992) indicated that residue A was a (1 → 4)-linked α-D-Glcp moiety. Predominant signal of 102.14 ppm, together with the typical signal of *O*-substituted C-4 at δ 79.27 ppm, supported the results from

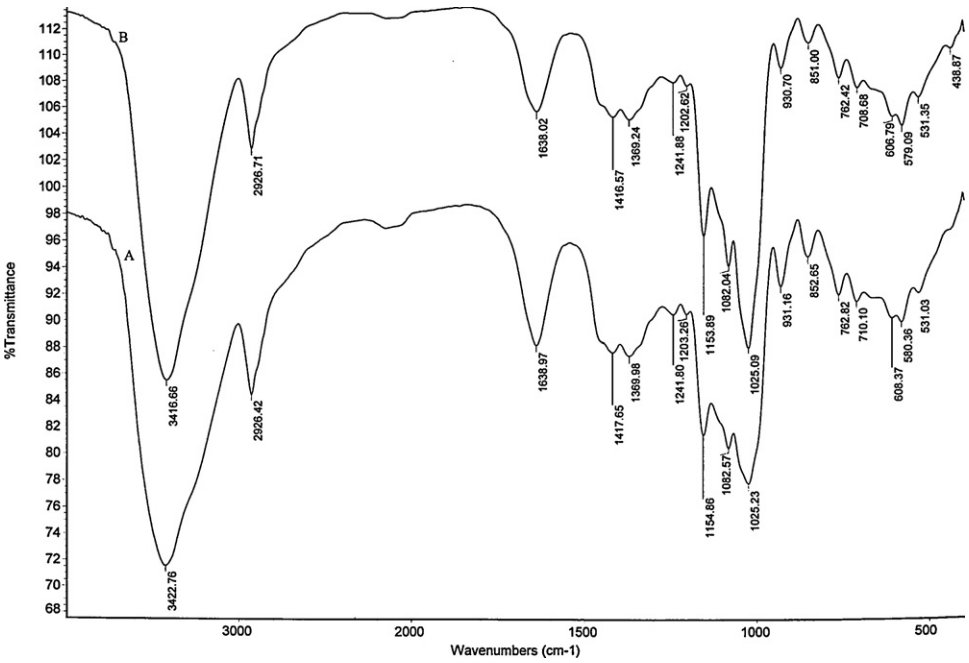

Fig. 3. FT-IR spectrum of SHG (A) and SHGp (B).

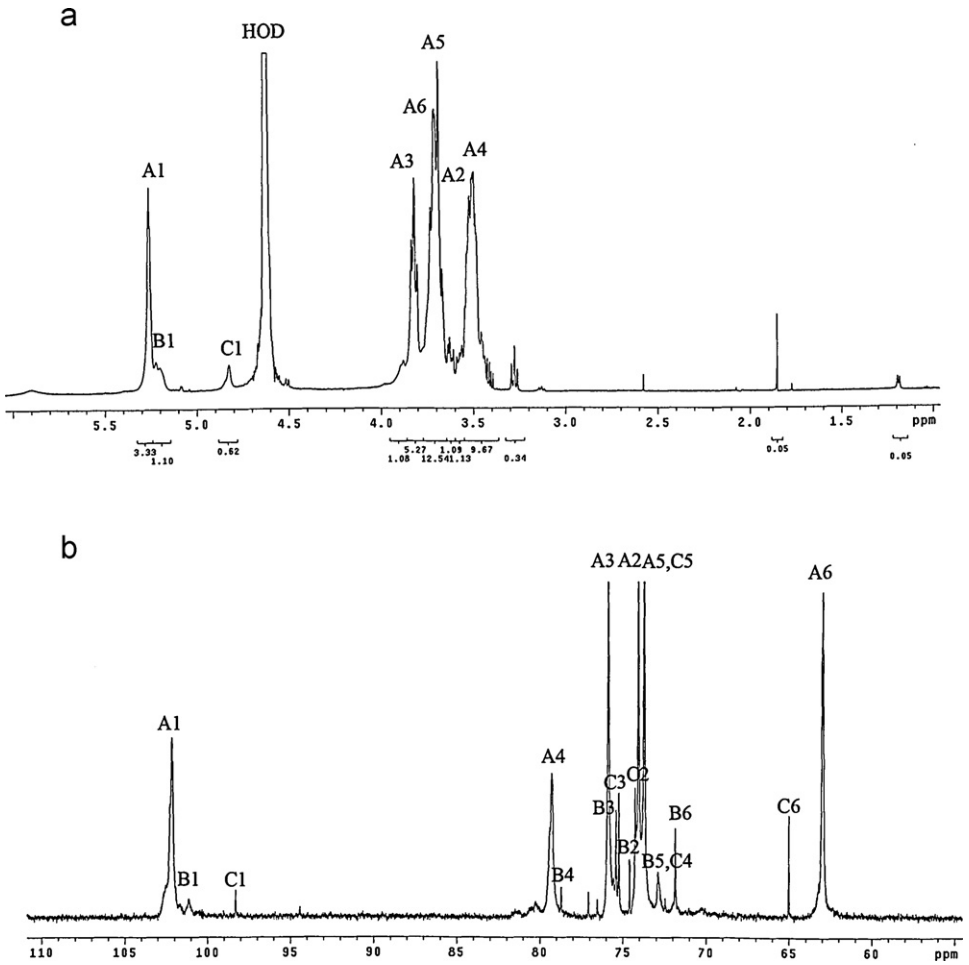

Fig. 4. (a) ^{1}H NMR spectrum (600 MHz, D_2O, 25 °C) of the sulfated glucan SHGp, isolated from the roots of *Hedysarum polybotrys*. (b) ^{13}C NMR spectrum (150 MHz, D_2O, 25 °C) of SHGp, isolated from the roots of *Hedysarum polybotrys*.

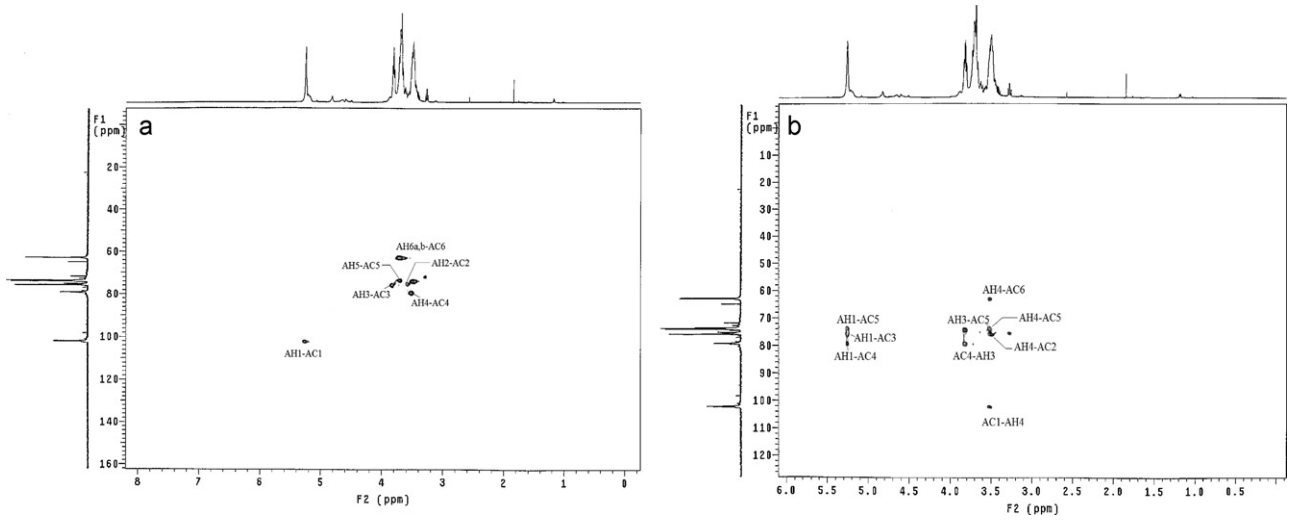

Fig. 5. (a) HSQC spectrum and (b) HMBC spectrum of SHGp, isolated from *Hedysarum polybotrys*.

chemical analysis, the high proportion of α-D-(1 → 4) linkages in the linear arrangement.

Residue B had an anomeric proton chemical shift at 5.21 ppm (H-1 resonances appeared as unresolved doublets), indicating that it was an α-linked residue. The split of H-1 may imply that the linkage is present as branch both terminal Glcp and sulfate group at *O*-substituted C-6. The ^{13}C signal for the anomeric carbon of the D-glucosyl moiety was observed at 101.11 ppm. The downfield shift of C-4 (78.70 ppm) and C-6 (71.84 ppm) signals with respect to the standard value of methyl glycosides was due to the effect of glycosylation (Seymour, Knapp, & Bishop, 1976). Hence, the carbon values of residue B indicate that it was α-D-(1 → 4,6)-linked glucose.

Residue C had an anomeric proton signal at δ 4.83 ppm indicate that it was an α-linked moiety. The C-1 signal of residue C at 98.29 ppm was confirmed according to evidence in the literature (Rout, Mondal, Chakraborty, & Islam, 2006; Roy et al., 2009). From the results of methylation analysis and NMR experiments, it could be concluded that C was terminal α-D-glucose.

The ^{1}H and ^{13}C signals of SHGp could be assigned using β-COSY, TOCSY, and HSQC NMR experiments. Although the presence of B and C was shown by the methylation analysis, no signal for B and C was identified in the 2D NMR spectra. The reason was that their low quantity did not allow detection in 2D NMR spectra and the other proton overlapping peaks (H2–H5) of SHGp were not assigned. Therefore, NMR is not appropriate to identify a small component even at substantial amounts.

The HSQC (Fig. 5(a)) spectrum of SHGp revealed information on correlation of ^{13}C and its linked ^{1}H: C-1/H-1 at δ 102.14/5.26 for (1 → 4)-α-D-glucosyl residues. C-6 (residue A: 62.94 ppm) correlated with two protons (residue A: δ 3.68, 3.64 ppm), C-2, C-3, C-4, C-5 each correlate with one proton. The atom signals of glucosyl ring carbon at δ 74.04, 75.86, 79.27, 73.68, 62.94 ppm corresponded, respectively, to C-2, C-3, C-4, C-5, C-6 of residue A.

The sequence of glycosyl residues of SHGp was determined by HMBC experiment (Fig. 5(b)). The cross peaks of both anomeric proton and carbon of each of the sugar moieties were examined, and intra- and inter-residual connectivities were observed. Cross peaks were observed between H-1 (δ 5.26) of residue A and C-4 (δ 79.27) of residue A (AH-1, AC-4); C-1 of residue A (δ 102.14) and H-4 (δ 3.45) of residue A. However, the cross peaks between H-6 of residue B and C-1 of residue C (BH-6, AC-1), C-6 of residue B and H-1 of residue C (BC-6, AH-1) were not observed due to the same reason as described. Nevertheless, (1 → 4,6)-linked D-glucose could confirm from ^{13}C NMR spectra. Unfortunately, the position of sulfate group in SHG did not be determined by NMR spectra. According Wang and Zhang (2009), the reactivities of the active hydroxyl groups were in the order of C-6 > C-4 > C-2 on the whole, combined with doublets of residue B, therefore, we deduce that the position of sulfate group could be at O-6.

Hence, based on all these chemical and spectroscopic evidences, it was confirmed that SHG had the structure of α-(1 → 4)-linked D-glucopyranosyl and terminated with one single terminal residue at the O-6 position of (1 → 4,6)-linked-α-D-glucopyranosyl approximately every eight residues, along the main chain. Sulfate groups may attach to the backbone at O-6, which one sulfate esters occasionally occur per thirty-eight anhydrous glucose units (Fig. 6).

So far, there is first reported on the sulfated glucan from *H. polybotrys*. However, a lot of glucans which have a fairly similar structure to SHG have been found in literature (de Lourdes Corradi da Silva et al., 2008; Fang & Wagner, 1988; Pang et al., 2007; Whistler et al., 1976; Yamada et al., 1986). This indicates that 1,4-linked α-D-glucan having side chains at O-6 of the glucosyl residues of the main chain are common in nature.

Sulfated polysaccharides commonly found in marine algae and higher animals but are absent in microbes and higher plants (Shanmugam & Mody, 2000). However, we isolated a sulfated glucan from *H. polybotrys*. Moreover, there is relatively little research pertaining to their biological activities of glucans mentioned above. Therefore, SHG is of special interest in the present study and we will concentrate on the biological activities.

In general, the biological activity of the sulfated polysaccharide is related to several structural parameters such as the degree of sulfation, the molecular weight, the sulfation pattern, glycosidic branchings, and type of sugar. The presence of the sulfate group is necessary for anti-HIV activity, and potency increase with increasing degree of sulfation (Schaeffer & Krylov, 2000). Sulfated homopolysaccharides, for example, are more potent as anti-HIV agents than heteropolysaccharides. Sulfation of the linear β-1,3-

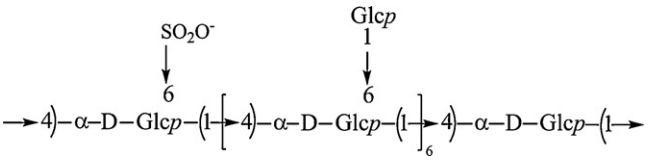

Fig. 6. The structure of SHG isolated from *Hedysarum polybotrys*. Sulfate groups may attach to the backbone at O-6, which one sulfate ester occasionally occur per 38 anhydrous glucose units.

glucan curdlan has been proven to result in potent anticoagulants in vitro, which exhibit antithrombotic activity in vivo comparable to that of heparin (Alban, Jeske, Welzel, Franz, & Fareed, 1995).

Sulfated polysaccharides are known to exhibit a wide range of physiological and biological activities, thus medically useful activities, such as antiviral (Lee, Hayashi, Maeda, & Hayashi, 2004), anticoagulant (Nishino & Nagumo, 1991), anti-tumor (Alekseyenko et al., 2007), anti-inflammatory (Kim, Kim, Shim, & Hwang, 2007), antioxidant (Chew, Lim, Omar, & Khoo, 2008).

To confirm whether SHG has biological activities, various biological activities for SHG, in connection with sulfated polysaccharides, should deserve more an in-depth research in the future, such as anti-HIV, anticoagulant, antioxidant activity. Further investigations on the relationship between biological activities and characteristic structure are presently underway in our laboratory.

4. Conclusions

Based on the data obtained, it could come to conclusion that a sulfated glucan (SHG) can be isolated from *H. polybotrys* in a very pure form by the method used in this study. Distinct from other reported polysaccharides from *H. polybotrys*, SHG possesses a small amount of sulfate groups. This is the first report on structure of sulfated glucan from *H. polybotrys*. Thus, structural analysis gives reliable results. Backbone chains of SHG are shown to be the linear 1,4-α-D-glucan, with a single 1,4,6-α-D-glucosyl side branching unit approximately every eight residues. Moreover, the branch is present as both terminal Glcp and sulfate group at O-6. The biological activities of SHG deserve more an in-depth research in the future.

Acknowledgments

This study was financed by a grant from Science and Technology Program of Gansu Province (no. 0911WCCA005). The authors are grateful to Professor Wei Zhou, for providing instrumental facilities of GC–MS, Gansu entry-exit inspection and quarantine bureau. The authors are also grateful to College of chemistry and chemical engineering of Lanzhou University for the NMR spectra and elemental analysis, Institute of Chemistry Chinese Academy of Sciences for running the SEC–MALLS experiments.

References

Agarwal, P. K. (1992). NMR spectroscopy in the structural elucidation of oligosaccharides and glycosides. *Phytochemistry*, *31*, 3307–3330.

Alban, S., Jeske, W., Welzel, D., Franz, G., & Fareed, J. (1995). Anticoagulant and antithrombotic actions of a semisynthetic beta-1,3-glucan sulfate. *Thrombosis Research*, *78*, 201–210.

Alekseyenko, T. V., Zhanayeva, S. Y., Venediktova, A. A., Zvyagintseva, T. N., Kuznetsova, T. A., Besednova, N. N., et al. (2007). Antitumor and antimetastatic activity of fucoidan, a sulfated polysaccharide isolated from the Okhotsk sea Fucus evanescens brown alga. *Bulletin of Experimental Biology and Medicine*, *147*, 730–732.

Barker, S. A., Bourne, E. J., Stacey, M., & Whiffen, D. H. (1954). Infra-red spectra of carbohydrates. Part I. Some derivatives of D-glucopyranose. *Journal of the Chemical Society. Chemical Communications*, 171–176.

Blumenkrantz, N., & Asboe-Hansen, G. (1973). New method for quantitative determination of uronic acids. *Analytical Biochemistry*, *54*, 484–489.

Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry*, *72*, 248–254.

Chakraborty, I., Mondal, S., Pramanik, M., Rout, D., & Islam, S. S. (2004). Structural investigation of a water-soluble glucan from an edible mushroom, *Astraeus hygrometricus*. *Carbohydrate Research*, *339*, 2249–2254.

Chew, Y. L., Lim, Y. Y., Omar, M., & Khoo, K. S. (2008). Antioxidant activity of three edible seaweeds from two areas in South East Asia. *LWT Food Science and Technology*, *41*, 1067–1072.

Chopin, T., & Whalen, E. (1993). A new and rapid method for carrageenan identification by FTIR diffuse-reflectance spectroscopy directly on dried, ground algal material. *Carbohydrate Research*, *246*, 51–59.

de Lourdes Corradi da Silva, M., Fukuda, E. K., Vasconcelos, A. F., Dekker, R. F., Matias, A. C., Monteiro, N. K., et al. (2008). Structural characterization of the cell wall D-glucans isolated from the mycelium of *Botryosphaeria rhodina* MAMB-05. *Carbohydrate Research*, *343*, 793–798.

Cui, H. X., Liu, Q., Tao, Y. Z., Zhang, H. F., Zhang, L. N., & Ding, K. (2008). Structure and chain conformation of a (1 → 6)-α-D-glucan from the root of *Pueraria lobata* (Willd.) Ohwi and the antioxidant activity of its sulfated derivative. *Carbohydrate Polymers*, *74*, 771–778.

Cui, X. M., Wang, Z. P., Zhang, Z. H., Ren, Y., Ma, J., & Huang, Z. L. (1998). Effect of radix hedysari polysaccharide (HPS) enhancing cytotoxicity of LAK cell on bladder cancer in vitro. *Pharmacology and Clinics of Chinese Materia Medica*, *14*, 19–20.

Delben, F., Forabosco, A., Guerrini, M., Liut, G., & Torri, G. (2006). Pullulans produced by strains of *Cryphonectria parasitica*—II. Nuclear magnetic resonance evidence. *Carbohydrate Polymers*, *63*, 545–554.

Doco, T., O'Neill, M. A., & Pellerin, P. (2001). Determination of the neutral and acidic glycosyl residue compositions of plant cell polysaccharides by GC–EI-MS analysis of the trimethylsilyl methyl glycoside derivatives. *Carbohydrate Polymers*, *46*, 249–259.

Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A., & Smith, F. (1956). Colorimetric method for determination of sugars and related substances. *Analytical Chemistry*, *28*, 350–356.

Fang, J. N., & Wagner, H. (1988). Chemical structure of a glucan from *Astragalus mongholicus*. *Acta Chimica Sinica*, *46*, 1101–1104.

Ghosh, K., Chandra, K., Ojha, A. K., & Islam, S. S. (2008). NMR and MALDI–TOF analysis of a water-soluble glucan from an edible mushroom, *Volvariella diplasia*. *Carbohydrate Research*, *343*, 2834–2840.

Hakomori, S. (1964). A rapid permethylation of glycolipid, and polysaccharide catalyzed by methylsulfinyl carbanion in dimethyl sulfoxide. *Journal of Biochemistry, Tokyo*, *55*, 205–208.

Hatanaka, K., Yoshida, T., Miyahara, S., Sato, T., Ono, F., Uryu, T., et al. (1987). Synthesis of new heparinoids with high anticoagulant activity. *Journal of Medicinal Chemistry*, *30*, 810–814.

Hu, F. D., Li, X. D., Zhao, L. G., Feng, S. L., & Wang, C. M. (2010). Antidiabetic properties of purified polysaccharide from *Hedysarum polybotrys*. *Canadian Journal of Physiology and Pharmacology*, *88*, 64–72.

Huang, Z. L., Cui, Z. M., Ren, Y., Zhang, J., Gan, M., Qi, W. X., et al. (1992). Empirical study of hedysari polysaccharide on anti-aging function. *Chinese Traditional and Herbal Drugs*, *23*, 469–473.

Jin, Z. S., Li, Y. D., Ru, Y. Q., Chu, H. Y., Wu, L. W., Ma, J., et al. (2004). Influence of hedysari polybotrys polysaccharide on nitric oxide, nitric oxide synthase, superoxide dismutase, and malondialdehyde of renal tissue in diabetic rat. *China Journal of Traditional Chinese Medicine and Western Medicine in Critical Care*, *11*, 141–144.

Jin, Z. S., & Zhang, D. P. (2006). Effects of radix hedysari on the contents of IL-6 in rats with insulin resistance of Type 2 diabetes mellitus. *Journal of Gansu College of Traditional Chinese Medicine*, *23*, 6–8.

Kim, A. J., Kim, Y. O., Shim, J. S., & Hwang, J. K. (2007). Immunostimulating activity of crude polysaccharide extract isolated from *Curcuma xanthorrhiza* Roxb. *Bioscience, Biotechnology, and Biochemistry*, *71*, 1428–1438.

Lahaye, M., & Yaphe, W. (1988). Effects of seasons on the chemical structure and gel strength of *Gracilaria pseudoverrucosa* agar (Gracilariales, Rhodophyta). *Carbohydrate Polymers*, *8*, 285–301.

Lan, Z. F., Zhang, Z. L., Cheng, G. Q., Wang, F. L., & Xi, S. F. (1987). Effects of radix hedysari polysaccharide on immunological function and transplanted tumors in mice. *Acta Pharmacological Sinica*, *8*, 275–277.

Lee, J. B., Hayashi, K., Maeda, M., & Hayashi, T. (2004). Antiherpetic activities of sulfated polysaccharides from green algae. *Planta Medica*, *70*, 813–817.

Li, B., Gu, X. H., & Xu, S. Y. (2004). Determination of the position of sulphate group in sulphated polysaccharides. *Journal of Analytical Science*, *20*, 498–500.

Li, S. G., Wang, D. G., Tian, W., Wang, X. X., Zhao, J. X., Liu, Z., et al. (2008). Characterization and anti-tumor activity of a polysaccharide from *Hedysarum polybotrys* Hand.-Mazz. *Carbohydrate Polymers*, *73*, 344–350.

Li, X. D., Hui, H. P., Feng, S. L., & OuYang, X. M. (2010). The process of removing proteins and depigmentation of polysaccharide from radix hedysari. *China modern medicine*, *17*, 40–41.

Ma, D., Feng, S. L., Zhang, L. G., Liu, X. H., Li, B., Li, X. D., et al. (2008). Purification of the radix hedysari polysaccharide and determination of its constitution. *Chinese Journal of Modern Applied Pharmacy*, *25*, 177–179.

Mahner, C., Lechner, M. D., & Nordmeier, E. (2001). Synthesis and characterization of dextran and pullulan sulphate. *Carbohydrate Research*, *331*, 203–208.

Mathlouthi, M., & Koenig, J. L. (1986). Vibrational spectra of carbohydrates. *Advances in Carbohydrate Chemistry and Biochemistry*, *44*, 7–89.

Melo, M. R. S., Feitosa, J. P. A., Freitas, A. L. P., & de Paula, R. C. M. (2002). Isolation and characterization of soluble sulfated polysaccharide from the red seaweed *Gracilaria cornea*. *Carbohydrate Polymers*, *49*, 491–498.

Nishino, T., & Nagumo, T. (1991). Change in the anticoagulant activity and composition of a fucan sulfate from the brown seaweed *Ecklonia kurome* during refrigerated storage of the fronds. *Botanica Marina*, *34*, 378–379.

Pang, X. B., Yao, W. B., Yang, X. B., Xie, C., Liu, D., Zhang, J., & Gao, X. D. (2007). Purification, characterization and biological activity on hepatocytes of a polysaccharide from *Flammulina velutipes* mycelium. *Carbohydrate Polymers*, *70*, 291–297.

Park, F. S. (1971). *Application of IR spectroscopy in biochemistry, biology, and medicine*. New York: Plenum Press.

Roslund, M. U., Tahtinen, P., Niemitz, M., & Sjoholm, R. (2008). Complete assignments of the ^{1}H and ^{13}C chemical shifts and $J_{H,H}$ coupling constants in NMR spectra of D-glucopyranose and all D-glucopyranosyl-D-glucopyranosides. *Carbohydrate Research*, *343*, 101–112.

Rout, D., Mondal, S., Chakraborty, I., & Islam, S. S. (2006). The structure of a polysaccharide from Fraction-II of an edible mushroom, *Pleurotus florida*. *Carbohydrate Research*, *341*, 995–1002.

Roy, S. K., Das, D., Mondal, S., Maiti, D., Bhunia, B., Maiti, T. K., et al. (2009). Structural studies of an immunoenhancing water-soluble glucan isolated from hot water extract of an edible mushroom, *Pleurotus florida*, cultivar Assam Florida. *Carbohydrate Research*, *344*, 2596–2601.

Schaeffer, D. J., & Krylov, V. S. (2000). Anti-HIV activity of extracts and compounds from algae and cyanobacteria. *Ecotoxicology and Environmental Safety*, *45*, 208–227.

Seymour, F. R., Knapp, R. D., & Bishop, S. H. (1976). Determination of the structure of dextran by ^{13}C nuclear magnetic resonance spectroscopy. *Carbohydrate Research*, *51*, 179–194.

Seymour, F. R., Knapp, R. D., Chen, E. C. M., Jeans, A., & Bishop, S. H. (1979). Structural analysis of dextrans containing 4-*O*-α-D-glucosylated α-D-glucopyranosyl residues at the branch points by use of ^{13}C nuclear magnetic resonance spectroscopy and gas liquid chromatography–mass spectrometry. *Carbohydrate Research*, *75*, 275–294.

Shanmugam, M., & Mody, K. H. (2000). Heparinoid-active sulfated polysaccharides from marine algae as potential blood anticoagulant agents. *Current Science*, *79*, 1672–1683.

Song, Q., Kobayashi, T., Xiu, L. M., Hong, T., & Cyong, J. C. (2000). Effects of Astragali root and Hedysari root on the murine B and T cell differentiation. *Journal of Ethnopharmacology*, *73*, 111–119.

Sweet, D. P., Shapiro, R. H., & Albersheim, P. (1975). Quantitative analysis by various GLC response-factor theories for partially methylated and partially ethylated alditol acetates. *Carbohydrate Research*, *40*, 217–225.

Tanaka, N., Sakamoto, G., Inoue, K., Korenaga, H., Ogawa, H., & Osada, Y. (1989). Antitumor effects of an antiangiogenic polysaccharide from an *Arthrobacter* species with or without a steroid. *Cancer Research*, *49*, 6727–6730.

Wang, J. G., & Zhang, L. N. (2009). Structure and chain conformation of five water-soluble derivatives of a β-D-glucan isolated from *Ganoderma lucidum*. *Carbohydrate Research*, *344*, 105–112.

Wang, J. Z., Ito, H., & Shimura, K. (1989). Enhancing effect of antitumor polysaccharide from astragalus or radix hedysarum on C3 cleavage production of macrophages in mice. *Japanese Journal of Pharmacology*, *51*, 432–434.

Whistler, R. L., Bushway, A. A., & Singh, P. P. (1976). Noncytotoxic antitumor polysaccharides. *Advances in Carbohydrate Chemistry and Biochemistry*, *32*, 235–275.

Yamada, H., Yanahira, S., Kiyohara, H., Cyong, J. C., & Otsuka, Y. (1986). Water-soluble glucan from the seed of *Coixlacryma-jobi* var. Ma-yuen. *Phytochemistry*, *25*, 129–132.

York, W. S., Darvill, A. G., McNeil, M., Stevenson, T. T., & Albersheim, P. (1986). Isolation and characterization of plant cell walls and cell wall components. *Method in Enzymology*, *118*, 3–40.

Zhang, Z. H., Yang, X. T., & Fang, J. N. (1997). Study on polysaccharide peptide with high performance gel permeation chromatography. *Chinese Journal of Chromatograph*, *15*, 150–152.

Zhou, Q., Jin, Z. S., Zhang, D. P., & Li, J. (2006). The effect of radix hedysari polysaccharide improve insulin resistance of type 2 diabetes mellitus rats with high-fat-feed. *Journal of Pediatrics of Traditional Chinese Medicine*, *2*, 24–27.