Sulfated modification, characterization and property of a water-insoluble polysaccharide from Ganoderma atrum

ARTICLE *in* INTERNATIONAL JOURNAL OF BIOLOGICAL MACROMOLECULES · MAY 2015

Impact Factor: 2.86 · DOI: 10.1016/j.ijbiomac.2015.04.070 · Source: PubMed

International Journal of Biological Macromolecules 79 (2015) 248–255

Contents lists available at ScienceDirect

International Journal of Biological Macromolecules

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

Sulfated modification, characterization and property of a water-insoluble polysaccharide from *Ganoderma atrum*

Hui Zhang[a,b], Jun-Qiao Wang[a], Shao-Ping Nie[a,*], Yuan-Xing Wang[a], Steve W. Cui[a,b], Ming-Yong Xie[a,*]

[a] State Key Laboratory of Food Science and Technology, Nanchang University, Nanchang, Jiangxi 330047, China
[b] Guelph Food Research Centre, Agriculture and Agri-Food Canada, Guelph, Ont., Canada N1G 5C9

ARTICLE INFO

Article history:
Received 24 February 2015
Received in revised form 25 April 2015
Accepted 27 April 2015
Available online 6 May 2015

Keywords:
Ganoderma atrum
Water-insoluble polysaccharide
Sulfated modification
Structure
Physicochemical property
Antioxidant activity

ABSTRACT

Sulfated modification was carried out to modify a water-insoluble polysaccharide from *Ganoderma atrum* (AGAP). The effects of sulfation on structure, physicochemical and functional properties of AGAP were investigated. Three sulfated derivatives were prepared, designated as S-1, S-2 and S-3 with degree of substitution (DS) of 0.35, 0.74 and 1.14, respectively. AGAP was elucidated as an α-(1→3)-glucan with few branches terminated by single mannose or xylose residues. The molecular weight (M_w) and radius of gyration (R_g) were estimated to be 1665 kDa and 65.49 nm, respectively. After sulfated modification, non-selective sulfation occurred preferably at *O*-6, partially at *O*-2 and *O*-4 positions of the glucosyl residues. The water-solubility of the derivatives was significantly improved in a DS-dependent manner. M_w of the derivatives showed a sharp decrease, and the chain conformation was estimated to be expanded stiff in phosphate buffer. *In vitro* tests showed that sulfated modification improved its antioxidant activities and anti-proliferative ability against S-180 tumor cells. This study suggested that sulfated modification was an effective approach to improve the water-solubility and functional properties of insoluble polysaccharides.

1. Introduction

The fungus of *Ganoderma* (*G.*) has been a popular edible mushroom in China, Japan, Korea and eastern Russia for more than 2000 years [1] and become the potentially important new source of anticancer agents in Western therapies [2]. In recent years, a water-extractable polysaccharide from the fruiting bodies of *Ganoderma atrum* (PSG-1) has been extensively studied and identified to possess significant immunomodulating and antitumor activities [3,4]. However, during the preparation of PSG-1, the fruiting body residues after water extraction were discarded, generating large amount of waste. As part of continuous effort to explore value-added products, a water-insoluble polysaccharide was extracted using alkaline solution from the water-extracted residues of *G. atrum*.

Unlike water-soluble polysaccharides, the water-insoluble polysaccharide attracts much less attention because of the poor water-solubility. In order to improve the water-solubility of polysaccharides, chemical modifications can be used by adding charge groups to the molecular chains. Sulfated modification is considered to be an effective, simple and rapid approach to modify polysaccharide structure. By inserting $-SO_3H$ groups to the free —OH position of sugar, the charge and electrostatic interaction of polysaccharide are increased, resulting in the enhancement of water solubility and conformation transition [5,6]. The sulfated polysaccharides have also been considered to possess better bioactivities than the native sample, such as antioxidant and antitumor activities, due to the polyelectrolyte form and improved solution property [7,8]. Several studies reported that sulfated modification could improve the solution and functional properties of the water-insoluble polysaccharides from mushrooms, *e.g. G. lucidum* and *Lentinus edodes* [9–11]. However, to our best knowledge, chemical modification on water-insoluble polysaccharide from *G. atrum* has not been reported.

Therefore, the objectives of present study were to modify the water-insoluble polysaccharide from *G. atrum* using chlorosulfonic acid-pyridine method and to investigate the effects of sulfated

* Corresponding authors. Tel.: +86 791 83969009; fax: +86 791 88304452.
E-mail addresses: zhh8672@126.com (H. Zhang), 815139617@qq.com (J.-Q. Wang), spnie@ncu.edu.cn, nie68@sina.com (S.-P. Nie), yuanxingwang@tom.com (Y.-X. Wang), Steve.Cui@agr.gc.ca (S.W. Cui), myxie@ncu.edu.cn (M.-Y. Xie).

http://dx.doi.org/10.1016/j.ijbiomac.2015.04.070
0141-8130/

modification on the structure, physicochemical and functional properties. The structures of the native and modified samples were analyzed using monosaccharide composition, FT-IR, ^{13}C NMR spectroscopy and high-performance size exclusion chromatography (HPSEC). 2,2-Diphenyl-1-picrylhydrazyl (DPPH) scavenging ability, Fe^{2+}-chelating capacity and anti-proliferation against S-180 tumor cells of sulfated derivatives were estimated.

2. Material and methods

2.1. Materials and chemicals

The fruiting bodies of *G. atrum* were purchased from Ganzhou, Jiangxi Province, China, which was harvested during the period of July 2011. All reagents used were of analytical grade unless otherwise specified.

2.2. Preparation and characterization of water-insoluble polysaccharide from G. atrum

The water-insoluble polysaccharide was extracted following the procedure outlined in Fig. 1. The water-insoluble fraction was further dissolved in 0.25 M LiCl/DMSO solution for 24 h. The polysaccharide precipitated during dialysis against water, which was collected by centrifugation and freeze dry, designated as AGAP. The sugar content of AGAP was determined by phenol–sulfuric acid method [12]. The homogeneity of AGAP in 0.5 M NaOH was detected by a size exclusion chromatography (SEC). Monosaccharide compositions were determined by a high-performance anion-exchange chromatography (HPAEC) according to previous report [13]. The polysaccharide (10 mg) was hydrolyzed by 2 M H_2SO_4 for 2 h at 100 °C, then injected onto the HPAEC system directly after dilution with water for 100 times. The linkage patterns were determined by methylation and GC–MS analysis according to the method of Ciucanu and Kerek [14] with minor modification as described in our previous report [15]. NMR spectroscopy including 1D (^1H, ^{13}C and DEPT-135 NMR) and 2D NMR (DQF-COSY, HSQC and NOESY) were conducted on a Bruker Avance 600 MHz NMR spectrometer (Brucker, Rheinstetten, Germany) at room temperature by dissolving sample in DMSO-d_6 to confirm the chemical shifts and linkage sequence of sugar residues.

2.3. Sulfated modification procedure of AGAP

Sulfated modifications of AGAP were prepared according to the procedure of chlorosulfuric acid (CSA)–pyridine method as described before [16] with minor modifications. The sulfating reagents with different ratio (CSA:Pyr = 1:1, 1:2, 1:4, v/v) were prepared. Samples (200 mg) were suspended in anhydrous formamide (20 mL) first and then transferred to a three-necked flask. The prepared sulfating reagents were added dropwise to the samples, the mixtures allowed to react at different temperatures and different reaction times (Table 1). After the reaction, the mixtures were cooled to room temperature and the pH value was adjusted to 7–8 with 1.0 mol/L NaOH solution. The sulfated polysaccharides were recovered after dialysis, precipitated with ethanol overnight and lyophilized. The sulfur contents of sulfated derivatives were determined by a barium chloride-gelatin nephelometry method [10]. The DS, which indicated the average number of sulfonic groups attached to a glucose unit, was calculated according to the follow equation [17]:

$$\mathrm{DS} = \frac{1.62 \times S\%}{32 - (1.02 \times S\%)} \quad (1)$$

where *S*% represented the sulfur content in the sample.

2.4. Structure characterizations of sulfated derivatives

The sugar contents were determined by phenol–sulfuric acid method at 490 nm as D-glucose equivalents [12]. The HPAEC procedure was conducted to study the effects of sulfated modification on monosaccharide composition of polysaccharide. An infrared absorption spectrum (FT-IR) and ^{13}C NMR spectroscopy of the sulfated derivatives were analyzed to confirm the sulfated modification. The FT-IR between 400 and 4000 cm^{-1} was recorded on a Thermo Nicolet 5700 infrared spectrophotometer (ThermoElectron, Madison, WI, USA). ^{13}C NMR was recorded following the same procedure as aforementioned by dissolving sulfated derivatives in D_2O.

2.5. Water solubility

The water solubility of sulfated polysaccharides was measured according to the method previously used for starch [18]. Each sample (100 mg) was suspended in 1.0 mL of ultra-pure water in a 5 mL micro-centrifuge tube. By dissolved at room temperature overnight with constant stirring, the solution was centrifuged at 12,000 rpm for 20 min. The un-dissolved residue was then separated from the supernatant and freeze dried. The water solubility was determined by the following equation:

$$\text{Water-solubility (g/L)} = \frac{m_o - m_p}{V} \quad (2)$$

where m_o is the mass of the original sample (mg), m_p is the mass of the precipitate after freeze dry (mg) and V is the volume of the water used to dissolve the sample (mL).

2.6. Molecular weight determination

The molecular weight (M_w) and radius of gyration (R_g) of AGAP in 0.5 M NaOH solution were measured by static light scattering (SLS) on a BI-200SM Brookhaven light scattering instrument at 633 nm (Brookhaven Instruments, New York, USA) according to a previous method [19]. d*n*/d*c* value of AGAP in 0.5 M NaOH solution was determined using BI-DNDC differential refractometer (Brookhaven Instruments Corporation) to be 0.138 mL/g. All the samples and solvent were filtered through 0.45 μm nylon filter for 4 times in order to remove dust. The concentrations of the tested sample were set at 0.0103–0.1030 mg/mL and the angles were from 40° to 140° at 5° interval. The M_w and the distribution of sulfated derivatives were carried out on an HPSEC with multiple detectors (Wyatt Technology Co., USA): a multi-angle laser light scattering, a differential pressure viscometer and a refractive index detector. The columns and detectors were maintained at 35 °C. The eluent was 0.25 mmol/L phosphate buffer (containing 0.02% NaN_3, pH 6.9) at a flow rate of 0.6 mL/min. Data were obtained and analyzed using ASTRA 6.1 software with a d*n*/d*c* value of 0.128 mL/g [20].

2.7. Assays for antioxidant activities

2.7.1. Assay of scavenging DPPH radicals

The free radical scavenging ability of samples was measured by DPPH test according to previous report [3]. Briefly, 1 mL of samples at different concentrations (0.125–8.0 mg/mL) was thoroughly mixed with 2 mL of freshly prepared DPPH (0.2 mM in 95% ethanol) and 2 mL of 95% ethanol. The mixture was shaken vigorously and allowed to stand for 30 min in dark and then the absorbance was measured at 517 nm. The capability to scavenge DPPH radicals was calculated using the following equation:

$$\text{Scavenging effect } \% = \frac{1 - (A_i - A_j)}{A_0} \times 100\% \quad (3)$$

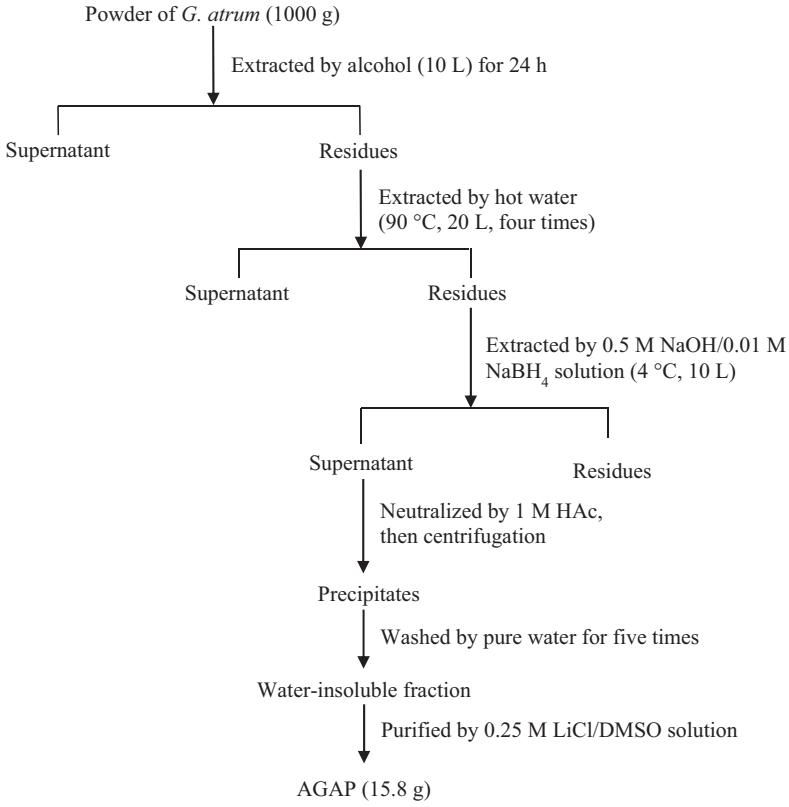


Fig. 1. Extraction procedure for the water-insoluble polysaccharide from *G. atrum* by cold 0.5 M NaOH/0.01 M $NaBH_4$ aqueous solution.

where A_0 is the absorbance of the DPPH solution without the sample, A_i is the absorbance of the test sample mixed with DPPH solution and A_j is the absorbance of the sample without the DPPH solution. Ascorbic acid (Vc) was used as a positive control.

2.7.2. Assay of chelating capacity on Fe^{2+}

The chelating capacity on Fe^{2+} of AGAP and sulfated derivatives was carried out according to previous reports [21] with minor modifications. Briefly, 1 mL of the samples at different concentrations (0.125–4.0 mg/mL) was mixed with 50 μL of $FeCl_2$ aqueous solution (2 mM) and 0.2 mL of ferrozine solution (5 mM), shaken well and allowed to stay still for 10 min at room temperature. EDTA was used as the positive control. The absorbance at 562 nm was used to calculate the chelating capacity according to the equation:

$$\text{Chelating capacity}\ (\%) = \frac{1-(A_1-A_2)}{A_0} \times 100\% \quad (4)$$

where A_0 is the absorbance of the control group without sample, A_1 is the absorbance of the test sample and A_2 is the absorbance of blank group without ferrozine.

2.8. Assay of anti-proliferative effect against S-180 tumor cells

The *in vitro* inhibition effects of sulfated derivatives on S-180 cell proliferation were evaluated using MTT assay. The cells in logarithmic growth phase were adjusted to a concentration of 5×10^4/mL using RPMI 1640 supplemented with 10% (v/v) heat-inactivated fetal bovine serum. 100 μL of the cell sample was pipetted into each well of 96-well plates and cultured for 4 h at 37 °C in 5% CO_2. Samples dissolved in RPMI 1640 (25–400 μg/mL) were added into the wells for determining its effect on cell growth. After following culture with samples for 48 h, 20 μL of MTT reagent (5 mg/mL) was added and the plate was read at 570 nm using a microplate reader (Thermo, Shanghai, China) after removal of the medium and dissolution of the dye crystals in DMSO. RPMI 1640 with 10% (v/v) heat-inactivated fetal bovine serum was used as solvent control. The inhibition rate was calculated according to the equation given below:

$$\text{Inhibition rate}\ (\%) = \frac{1-(A_{\text{sample}}-A_{\text{blank}})}{A_{\text{control}}} \times 100\% \quad (5)$$

where A_{control} is the absorbance of the control group without sample, A_{sample} is the absorbance of the test sample and A_{blank} is the

Table 1
The sulfated conditions, degree of substitution (DS), yield and sugar content of AGAP and sulfated derivatives.

Sample	CSA:Pyr (v/v)	Temp. (°C)	Time (h)	DS	Yield (w/w, %)	Sugar content (%)[a]
AGAP	–	–	–	n.d.[b]	1.58[c]	98.9 ± 0.20
S-1	1:4	45	3	0.35	86.5[d]	66.0 ± 0.36
S-2	1:1	75	4	0.74	105.6[d]	59.0 ± 0.29
S-3	1:2	60	2	1.14	108.1[d]	67.0 ± 0.74

[a] Data were shown as mean ± SD, $n = 3$.
[b] n.d.: not detected.
[c] Calculated based on the weight of extracts to the dry mass of *G. atrum* residues.
[d] Calculated based on the weight of derivatives to the weight of native polysaccharide samples.

absorbance of blank group without cells. The experiment was carried out in quintuplicate and averaged.

2.9. Statistical analysis

All the data were calculated with three replications and shown in mean ± standard deviation (SD), with significance $P < 0.05$ after subjecting to an analysis of variance (ANOVA) by Origin V8.0 (OriginLab Corp., Northampton, MA, USA).

3. Results and discussion

3.1. Sulfated modification of AGAP

The yield of AGAP extracted by 0.5 M NaOH aqueous solution at 4 °C was 1.58% from dry fungal mass. The sugar content of AGAP was as high as 98.9% and the sulfur content test was negative (Table 1). The eluted curve of AGAP on a Hiload Superdex-200 prep grade column showed to be a single peak by using 0.1 M NaOH solution as eluent, indicating that AGAP was homogeneous (Fig. S1). When polysaccharides are subjected to the sulfation reaction, some of the hydroxyl groups in the chains will be replaced by $-SO_3H$ group. This substitution reaction happens in variable degrees according to different reaction conditions. The sulfated modification of AGAP followed an orthogonal design test to obtain sulfated derivatives with different DS. Three sulfated derivatives with yield of 86.5–108.1% were chosen for further studies and designated as S-1, S-2 and S-3 with DS of 0.35, 0.74 and 1.14, respectively (Table 1). The sugar content decreased in all the sulfated derivatives as shown in Table 1, which is in accordance with previous studies [16,22]. The reasons for this phenomenon could be explained as follows: (1) the insertion of $-SO_3H$ group into the glucosyl residue increases the molecular weight of the residue. Theoretically, the sugar content of the polysaccharide will decrease to some extent; (2) the insertion of $-SO_3H$ into the glucosyl residue would affect the colorimetric reaction, leading to the low absorbance of tested samples, finally resulting in the decrease of the determined sugar content. However, the real reasons for this problem are still not clear and need further studies.

3.2. Structure characterizations of AGAP and sulfated derivatives

Monosaccharide composition analysis revealed that the predominant sugar residue of AGAP was glucose (87.68%), with small amount of mannose (6.14%) and xylose (4.45%). The results indicated that AGAP might be a heteroglucan (Table 2). Methylation analysis indicated that 1,3-linked-Glc*p* was the major linkage pattern of AGAP, which accounted for 86.54% of all sugar residues based on peak area. The mannose and xylose were found to be terminal residues, which were linked with 1,3,6-linked-Glc*p* residue (6.64%). After sulfated modification, the monosaccharide composition had no significant change. The main sugar residue was still glucose with a little higher percentage, while the terminal residues of mannose and xylose in the side chain decreased to some extent in all three derivatives (Table 2). The minor differences might be due to the slight removal of side chains in the derivatives.

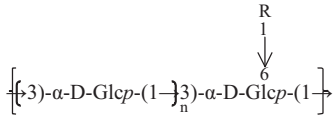
Table 2
The monosaccharide composition analysis of AGAP and sulfated derivatives.[a]

Monosaccharide composition (%)			
Sample code	Glc	Man	Xyl
AGAP	87.68	6.14	4.45
S-1	92.50	5.25	2.25
S-2	91.62	6.29	2.09
S-3	93.54	5.08	1.38

[a] Calculated as relative percentage of all sugar present, based on the standard curves of each monosaccharide.

The FT-IR spectra of AGAP and the sulfated derivatives are shown in Fig. 2. All the spectra exhibited typical absorption peaks assigned to the saccharide matrix. The peak of 3421 cm^{-1} was the —OH stretching vibration and the band in the region of 2921 cm^{-1} was the C—H stretching vibration. The absorption at around 1660 cm^{-1} was attributed to the H—O—H angle vibration of bond water [23]. The different intensity of this peak indicated the different water-absorption capacity of samples. The absorptions at 930, 847 and 822 cm^{-1} appearing in the AGAP indicated that (1→3)-D-glucose was in the α-form; especially, the peak at 822 cm^{-1} is exclusively associated with the α-(1→3)-linkage [24]. After sulfated modification, two new characteristic absorption bands at the vicinity of 1260 and 820 cm^{-1} appeared in sulfated derivatives due to the S=O stretching vibration and C—O—S vibration, respectively [9], indicating that the sulfonic groups were successfully introduced onto the AGAP molecules. The different intensity of S-1 to S-3 at 820 cm^{-1} revealed the different content of sulfonic groups in the sulfated derivatives.

NMR spectra were used to investigate the structural features of AGAP and its sulfated derivatives. The ^{13}C NMR of AGAP (151.01 MHz, Fig. 3A) showed six carbon signals with approximately equal intensity, which is typical of α-(1→3)-glucan [25,26]. DEPT-135 spectrum (Fig. 3B) and 2D NMR (DQF-COSY, HSQC and NOESY, Fig. S2) were further used to confirm the chemical shifts of all the carbons and protons of (1→3)-glucosyl residues in AGAP (Table 3). However, the small amount of T-Xyl*p*, T-Man*p* and 1,3,6-linked-Glc*p* residues were not detected by NMR spectroscopy in this study due to the low sensitivity of ^{13}C NMR. Even so, based on all the chemical and spectroscopic data, it could be deduced that AGAP was a heteroglucan consisting of α-(1→3)-glucan backbone, with few branches at *O*-6 position of glucosyl residues terminated by mannosyl or xylosyl residues. A possible structural fragment of AGAP is proposed as following:



where R would be either T-D-Man*p* or T-D-Xyl*p* residue; the number of α-(1→3)-glucosyl residues in the fragment (n) would be 13 according to methylation results.

The ^{13}C NMR spectra of sulfated derivatives were shown in Fig. 3C–E to study the substituted position of sulfation. By comparing with Fig. 3A, more complicated spectra were observed due to the introduction of electronegative sulfonic ester groups. Briefly, the signals of C-6 shifted from δ 60.75 ppm in AGAP to low field (δ 66.25, 65.73 and 65.87 ppm for S-1, S-2 and S-3, respectively), indicating that the C-6 hydroxyl groups were sulfated completely. Two new signals at δ 78.67 ppm (C2s) and δ 76.48 ppm (C4s) appeared in S-3, indicating that the sulfated modification of the hydroxyl groups partially took place at C-2 and C-4 positions [9,27]. However, no or very low signal of these two peaks appeared in S-1 and S-2, revealing that the degree of substitution at C-2 and/or C-4 positions was very low. Additionally, the low intensity of anomeric signal (C-1′) at around δ 103.40 ppm in S-3 was due to the substitution at C-2 which would influence the chemical shift of the adjacent C-1 (Table 3). The downfield shift of C-1′ to C-1 was about 3 ppm, which is consistent with the results of sulfated κ-carrageenan [28]. Based on the results of ^{13}C NMR and the structure of AGAP, the non-selective sulfation reaction was identified to occur preferably at *O*-6, partially at *O*-2 and *O*-4 position of the glucosyl residues. Lower steric hindrance of C-6 in α-(1→3)-glucan was considered to be the main reason for the priority of substitution [11,29].

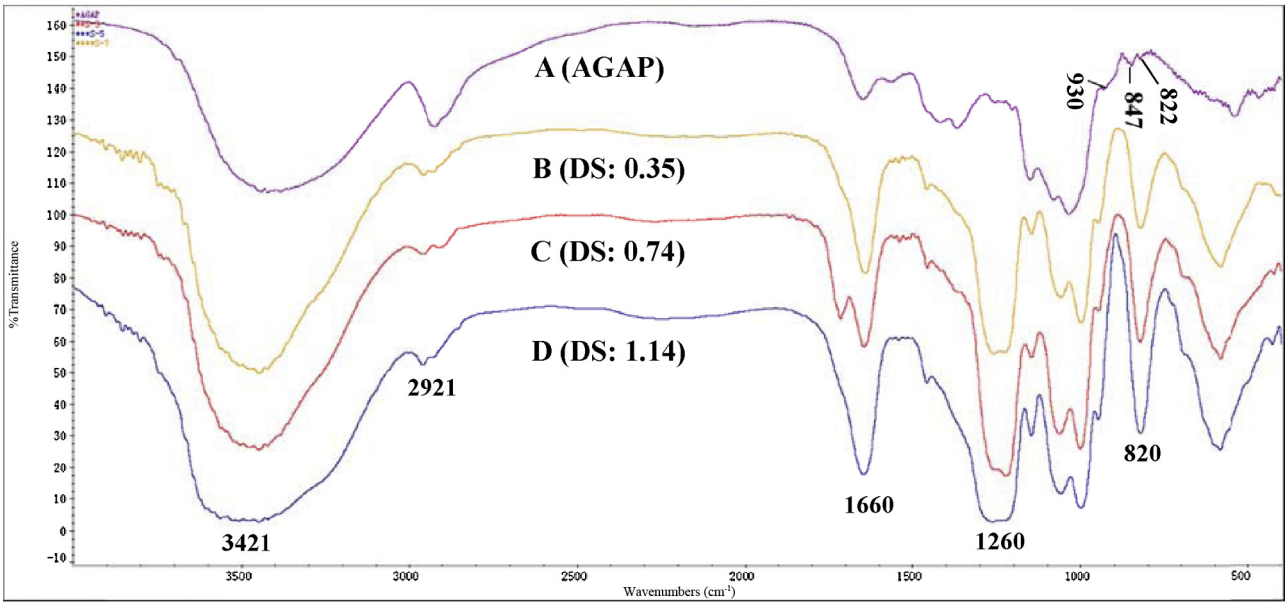


Fig. 2. FT-IR spectra of AGAP and its sulfated derivatives with different DS values: (A) the native sample of AGAP; (B–D) the sulfated derivatives of S-1, S-2 and S-3 with different DS values, respectively.

3.3. Analysis of physicochemical properties

Good water-solubility is very important for polysaccharide to exert its functional properties in medicinal and food applications. Water-solubility test showed that the native polysaccharide (AGAP) was insoluble (suspending in water), while sulfated modification significantly increased its solubility. Results from Table 4 showed that the soluble mass of S-1, S-2 and S-3 in 1 L of water was 11.2, 66.3 and 76.5 g, respectively, presenting in a DS-dependent manner. This observation is in accordance with previous reports [9,30].

The molecular parameters of AGAP were determined by SLS method in 0.5 M NaOH solution. The results from the Berry-plot of AGAP showed that M_w and R_g were 1665 kDa and 65.49 nm, respectively (Table 4). The M_w, R_g and intrinsic viscosity ([η]) of water-soluble sulfated derivatives were measured by HPSEC with multiple online detectors and are summarized in Table 4. The M_w of S-1, S-2 and S-3 were 232.9, 20.97 and 484.8 kDa, respectively. The intrinsic viscosity of the sulfated derivatives showed a positive correlation with the M_w, which is in accordance with previous studies [31]. By comparing with the native sample of AGAP, all the derivatives showed a sharp decrease in molecular weight. Similar findings were also observed from other studies [8,16], the reasons were ascribed to the extensive degradation of the polymer during the reaction triggered by hydrolysis of polysaccharide in acid environment. The low M_w of S-2 might be due to the high reaction temperature and long reaction time.

Using HPSEC combined with online viscometer, the double logarithmic plot of the molecular weight *vs* intrinsic viscosity can be well described using the Mark–Houwink equation:

$$[\eta] = kM_w^{\alpha} \quad (6)$$

where k and α are used to estimate the corresponding conformation of polysaccharide. The exponent (α) is related to the shape and chain structure of the polymer as well as to the nature of the solvent. In general, for a flexible random coil polymer in a good solvent, α value is in the range of 0.5–0.8. When the polymer chain is more extended, or presents as semi-stiff, the α value is above 0.8 and even beyond 1.0 [19,32]. The Mark–Houwink equations of sulfated derivatives are shown in Table 4. The α values of S-1, S-2 and S-3 were obtained as 1.12, 0.93 and 1.37, respectively, indicating that the sulfated derivatives exhibited expanded stiff chain conformation in phosphate buffer. The stiff conformation of the sulfated derivatives might be due to the insertion of $-SO_3H$ groups, which could increase the charge density and electrostatic repulsion of polysaccharide molecules [8].

3.4. Antioxidant activity analysis

Glucans were considered to be weak in exerting free radical scavenging and antioxidant ability. However, polyelectrolytes, such as sulfated or phosphorylated glucans and lipopolysaccharide, showed considerably higher scavenging capacity [33]. In the current study, the antioxidant activities of AGAP and the sulfated derivatives were evaluated *via* DPPH radical scavenging and Fe^{2+} chelating capacities assays to estimate the effects of sulfated modification on the antioxidant activity.

As shown in Fig. 4, all the samples were found to possess the DPPH radical scavenging ability and chelating capacity on ferrous ions in dose-dependent manner, but the sulfated derivatives showed a significant increase by comparing to AGAP. The scavenging rate and chelating ability of the maximum concentration of AGAP were less than 10% and the IC_{50} value was not calculated. After sulfated modification, the IC_{50} values for DPPH

Table 3
The ^{13}C NMR signal assignments (in ppm) of AGAP and its sulfated derivatives.

Sample	C1	C1′	C2	C3	C4	C5	C6	C2s	C4s	C6s
AGAP	100.37	–	71.41	83.34	69.92	72.49	60.64	–	–	–
S-1	100.94	–	71.77	82.71	68.75	73.85	–	–	–	66.25
S-2	101.03	–	71.86	82.82	68.84	73.96	–	–	–	65.73
S-3	100.34	103.40	71.18	79.11	69.42	72.40	–	78.67	76.48	65.87

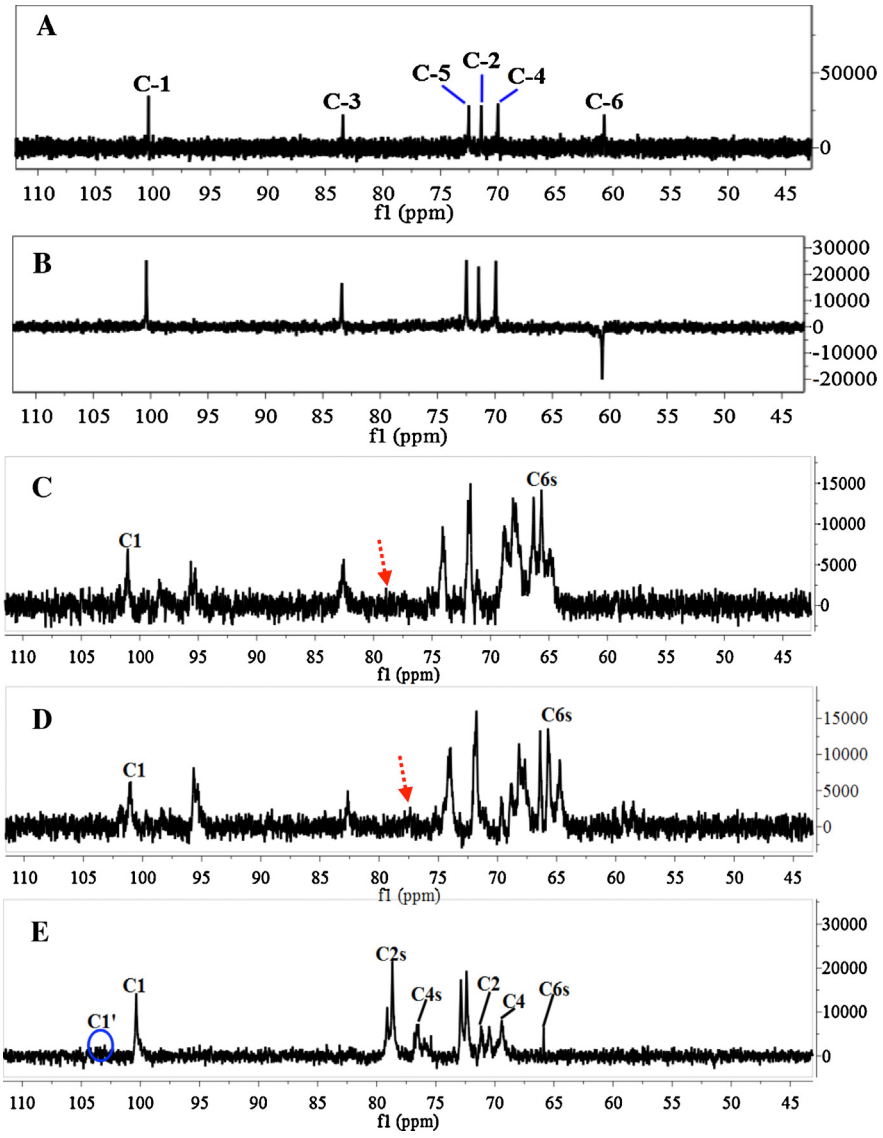


Fig. 3. The NMR spectra of AGAP and sulfated derivatives with different DS values (recorded at 294 K). (A, B) the ^{13}C (151.01 MHz) and DEPT-135 NMR spectrum of AGAP dissolved in DMSO-d_6, respectively; (C–E) the ^{13}C (151.01 MHz) spectrum of S-1, S-2 and S-3 dissolved in D_2O, respectively.

scavenging and Fe^{2+} chelating capacities decreased significantly. The IC_{50} values for DPPH scavenging ability were 15.82, 15.88 and 1.80 mg/mL of S-1, S-2 and S-3, respectively. And the IC_{50} values for Fe^{2+}-chelating capacities of S-1, S-2 and S-3 were 3.49, 5.87 and 1.90 mg/mL, respectively. These results indicated that the insertion of $-SO_3H$ groups had noticeable effects on the abilities of scavenging DPPH free radicals and chelating metal ions. The different IC_{50} values of the sulfated derivatives indicated that S-3 with the highest DS value and most expanded stiff chain possessed the best antioxidant activities. However, the correlation among activity, structure and physicochemical properties was not clearly investigated in this study and needed further discussion.

3.5. Anti-proliferative activity against S-180 tumor cells

The anti-proliferative effect of sulfated derivatives on S-180 tumor cell line was investigated using MTT assay. As shown in Fig. 5, all the sulfated derivatives inhibited the *in vitro* proliferation

Table 4
The molecular parameters of AGAP and its sulfated derivatives.

Sample	Water-solubility (g/L)	M_w (kDa)	R_g (nm)	M_w/M_n (PI)	$[\eta]$ (dL/g)	Mark–Houwink equation[d]
AGAP[a]	n.d.[c]	1665	65.49	–	–	–
S-1[b]	11.2 ± 0.94	232.9	45.7	3.7	2.55	$[\eta] = 4.0 \times 10^{-3} M_w^{1.12}$
S-2[b]	66.3 ± 1.68	20.97	17.2	1.94	0.71	$[\eta] = 3.40 \times 10^{-3} M_w^{0.93}$
S-3[b]	76.5 ± 2.10	484.8	62.3	4.1	3.34	$[\eta] = 1.48 \times 10^{-5} M_w^{1.37}$

[a] The data were from the Berry-plots of AGAP, which was dissolved in 0.5 M NaOH solution, using static light scattering method.
[b] The data were from the results of HPSEC and analyzed using ASTRA 6.1 software. The sulfated derivatives were dissolved in 0.25 mmol/L phosphate buffer (containing 0.02% NaN_3, pH 6.9).
[c] n.d.: not detected.
[d] The Mark–Houwink equations were obtained from the good linear fit based on the cumulative weight fraction which possessed 90% of the total weight.

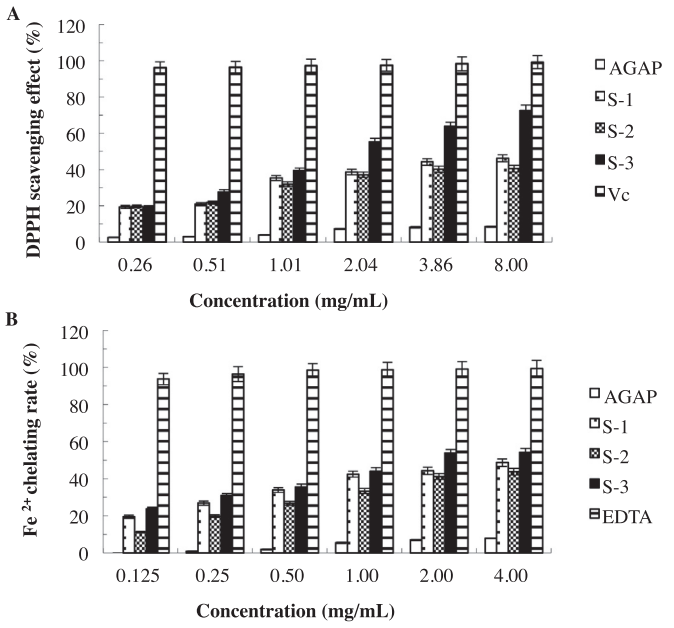


Fig. 4. The *in vitro* antioxidant of AGAP and its sulfated derivatives. (A) the scavenging ability against DPPH radicals; (B) the chelating capacity on ferrous ions. All measurements were conducted in triplicate, and mean values are reported. The vertical bars represent the standard deviation of each data point.

of S-180 tumor cells in a dose-dependent manner. The IC_{50} values of S-1, S-2 and S-3 were 0.99, 0.76 and 0.12 mg/mL, respectively. The results indicated that the anti-proliferative activity of the sulfated derivatives was in positive correlation with the DS value, rather than the molecular weight. Unfortunately, due to the water-insoluble property, the native sample of AGAP could not be used for the *in vitro* test in the same condition.

The anti-proliferative effect of sulfated derivatives on S-180 tumor cell was compared with a water-soluble polysaccharide from *G. atrum* (PSG-1) which had been reported before [34]. PSG-1 was found to possess no direct anti-proliferative effect on S-180 tumor cell *in vitro*. Its antitumor activity was exerted *via* activating the host immune function of tumor-bearing mice. However, the sulfated derivatives of AGAP could inhibit the proliferation of S-180 tumor cells directly. Previous studies also reported that sulfated polysaccharides could inhibit the proliferation of HepG2 liver cancer cells and reduce the *in vivo* growth of S-180 solid tumor [8]. The introduction of sulfated groups was considered to be crucial to the enhancement of the antitumor activities. In view of the results mentioned above, the sulfated derivatives of AGAP with high DS value would be a potential antitumor agent. The *in vivo* antitumor activities and the possible mechanism will be discussed in our further work.

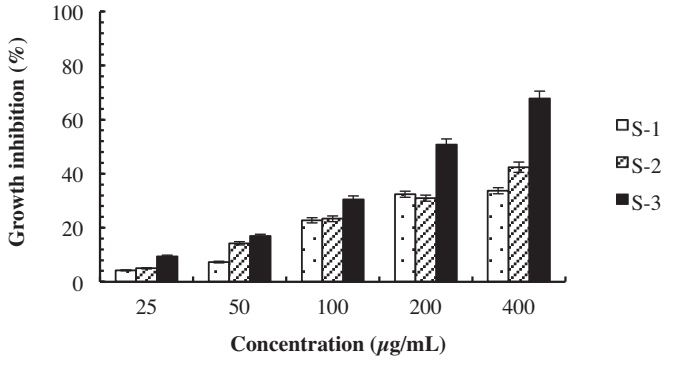


Fig. 5. The *in vitro* anti-proliferative effects against S-180 tumor cells of sulfated derivatives with different DS values. All measurements were conducted in quintuplicate, and mean values are reported. The vertical bars represent the standard deviation of each data point.

4. Conclusion

A water-insoluble by-product from the water-extracted residues of *G. atrum* (AGAP) was characterized to be α-(1→3)-glucan with few branches at *O*-6 positions terminated by mannose or xylose residues. Sulfated modification was applied to modify the molecular structure in the purpose of improving water-solubility. Three sulfated derivatives with different DS value were prepared. The water-solubility of the derivatives was significantly improved in a DS-dependence manner. All the derivatives had a sharp decrease in molecular weight comparing to AGAP. Solution property analysis based on Mark–Houwink equation revealed that sulfated AGAP exhibited expanded stiff chain in phosphate buffer. *In vitro* tests showed that sulfated derivatives exhibited better antioxidant activity than the native sample. The anti-proliferative activity against S-180 tumor cells was also improved in positive correlation with the DS value. The improved physicochemical and functional properties of the sulfated derivatives would be somewhat beneficial for the food and medicinal utilization of AGAP and for the economic value of *G. atrum* mushroom.

Acknowledgements

The financial support for this study by the National Natural Science Foundation of China (Nos. 21265011, 31160321), Key Program of National Natural Science Foundation of China (No. 31130041), the Program for New Century Excellent Talents in University (NCET-12-0749) and Special Fund of Jiangxi Province for the Innovation of Post-graduates (YC2012-B003) is gratefully acknowledged. The authors also thank Dr. Qingbin Guo (University of Guelph, Canada) for the helpful discussion on HPSEC and conformation analysis.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ijbiomac.2015.04.070

References

[1] R.R.M. Paterson, Phytochemistry 67 (2006) 1985–2001.
[2] R. Sullivan, J.E. Smith, N.J. Rowan, Perspect. Biol. Med. 49 (2006) 159–170.
[3] Y. Chen, M.Y. Xie, S.P. Nie, C. Li, Y.X. Wang, Food Chem. 107 (2008) 231–241.
[4] W.J. Li, S.P. Nie, Y. Chen, Y.X. Wang, C. Li, M.Y. Xie, J. Agric. Food Chem. 59 (2011) 3707–3716.
[5] R. Jayakumar, N. Nwe, S. Tokura, H. Tamura, Int. J. Biol. Macromol. 40 (2007) 175–181.
[6] C. Mähner, M.D. Lechner, E. Nordmeier, Carbohydr. Res. 331 (2001) 203–208.
[7] S.K. Chen, M.L. Tsai, J.R. Huang, R.H. Chen, J. Agric. Food Chem. 57 (2009) 2699–2704.
[8] Y.Z. Tao, L.N. Zhang, P.C.K. Cheung, Carbohydr. Res. 341 (2006) 2261–2269.
[9] X.F. Bao, J.Y. Duan, X.Y. Fang, J.N. Fang, Carbohydr. Res. 336 (2001) 127–140.
[10] W. Liu, H.Y. Wang, W.B. Yao, X.D. Gao, L.L. Yu, J. Agric. Food Chem. 58 (2010) 3336–3341.
[11] P.Y. Zhang, P.C.K. Cheung, Biosci. Biotechnol. Biochem. 66 (2002) 1052–1056.
[12] M. DuBois, K.A. Gilles, J.K. Hamilton, P.A. Rebers, F. Smith, Anal. Chem. 28 (1956) 350–356.
[13] Q. Guo, S.W. Cui, Q. Wang, X. Hu, Q. Guo, J. Kang, R. Yada, Carbohydr. Polym. 86 (2011) 831–836.
[14] I. Ciucanu, F. Kerek, Carbohydr. Res. 131 (1984) 209–217.
[15] H. Zhang, W.J. Li, S.P. Nie, Y. Chen, Y.X. Wang, M.Y. Xie, Carbohydr. Polym. 88 (2012) 1047–1054.
[16] J. Wang, H. Guo, J. Zhang, X. Wang, B. Zhao, J. Yao, Y. Wang, Carbohydr. Polym. 81 (2010) 897–905.
[17] W. Zhang, Biochemical Technology of Study on Carbohydrate Complex, 2nd ed., Zhejiang University Press, Hangzhou, 1999.

[18] R. Mukerjea, G. Slocum, J.F. Robyt, Carbohydr. Res. 342 (2007) 103–110.
[19] Q. Guo, Q. Wang, S.W. Cui, J. Kang, X. Hu, X. Xing, R.Y. Yada, Food Hydrocoll. 32 (2013) 155–161.
[20] L.N. Zhang, M. Zhang, Q. Zhou, J.H. Chen, F.B. Zeng, Biosci. Biotechnol. Biochem. 64 (2000) 2172–2178.
[21] W. Raza, K. Makeen, Y. Wang, Y.C. Xu, Q.R. Shen, Bioresour. Technol. 102 (2011) 6095–6103.
[22] Y. Sun, H. Liang, G. Cai, S. Guan, H. Tong, X. Yang, J. Liu, Int. J. Biol. Macromol. 44 (2009) 14–17.
[23] F.S. Parker, Applications of Infrared Spectroscopy in Biochemistry, Biology and Medicine, Plenum Press, New York, 1971.
[24] F.R. Seymour, R.L. Julian, A. Jeanes, B.L. Lamberts, Carbohydr. Res. 86 (1980) 227–246.
[25] E.R. Carbonero, A.V. Montai, S.M. Woranovicz-Barreira, P.A. Gorin, M. Iacomini, Phytochemistry 61 (2002) 681–686.
[26] A. Choma, A. Wiater, I. Komaniecka, R. Paduch, M. Pleszczyńska, J. Szczodrak, Carbohydr. Polym. 91 (2013) 603–608.
[27] A. Gamzazade, A. Sklyar, S. Nasibov, I. Sushkov, A. Shashkov, Y. Knirel, Carbohydr. Polym. 34 (1997) 113–116.
[28] P.A. Gorin, Adv. Carbohydr. Chem. Biochem. 38 (1981) 13–104.
[29] K. Katsuraya, H. Nakashima, N. Yamamoto, T. Uryu, Carbohydr. Res. 315 (1999) 234–242.
[30] H.Y. Jung, I.Y. Bae, S.Y. Lee, H.G. Lee, Food Hydrocoll. 25 (2011) 1291–1295.
[31] Y. Lin, L. Zhang, L. Chen, Y. Jin, F. Zeng, J. Jin, B. Wan, P.C.K. Cheung, Int. J. Biol. Macromol. 34 (2004) 231–236.
[32] Y.Z. Tao, L.N. Zhang, F. Yan, X.J. Wu, Biomacromolecules 8 (2007) 2321–2328.
[33] E. Tsiapali, S. Whaley, J. Kalbfleisch, H.E. Ensley, I.W. Browder, D.L. Williams, Free Radical Biol. Med. 30 (2001) 393–402.
[34] S. Zhang, S. Nie, D. Huang, J. Huang, Y. Feng, M. Xie, Food Chem. Toxicol. 68 (2014) 239–246.