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# Regioselective sulfation of *Artemisia sphaerocephala* polysaccharide: Solution conformation and antioxidant activities in vitro

Junlong Wang[a,b,*], Wen Yang[a,b], YinYing Tang[a,b], Qing Xu[a,b], Shengli Huang[a,b], Jian Yao[a,b], Ji Zhang[a,b,c], Ziqiang Lei[c]

[a] College of Life Science, Northwest Normal University, Lanzhou 730070, People's Republic of China
[b] Bioactive Products Engineering Research Center For Gansu Distinctive Plants, Northwest Normal University, Lanzhou 730070, People's Republic of China
[c] Key Laboratory of Eco-Environment-Related Polymer Materials Ministry of Education, College of Chemistry and Chemical Engineering, Northwest Normal University, Lanzhou 730070, People's Republic of China

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ABSTRACT

Regioselective modification is an effective approach to synthesize polysaccharides with different structure features and improved properties. In this study, regioselective sulfation of *Artemisia sphaerocephala* polysaccharide ($S_{RS}$ASP) was prepared by using triphenylchloromethane (TrCl) as protecting precursor. The decrease in fractal dimension ($d_f$) values (1.56–2.04) of $S_{RS}$ASP was observed in size-exclusion chromatography combined with multi angle laser light scattering (SEC-MALLS) analysis. Compared to sample substituted at C-6, $S_{RS}$ASP showed a more expanded conformation of random coil, which was attributed to the breakup of hydrogen bonds and elastic contributions. Circular dichroism (CD), methylene blue (MB) and congo red (CR) spectrophotometric method and atomic force microscopy (AFM) results confirmed the conformational transition and stiffness of the chains after sulfation. $S_{RS}$ASP exhibited enhanced antioxidant activities in the DPPH, superoxide and hydroxyl radical scavenging assay. Sulfation at C-2 or C-3 was favorable for the chelation which might prevent the generation of hydroxyl radicals. It concluded that the degree of substitution and substitution position were the factors influencing biological activities of sulfated polysaccharides.



## 1. Introduction

It is generally admitted that the biological activities of polysaccharides are dependent on the structure features, such as the compositions, average molecular weight, degree of substitution (DS), substitution positions and chain conformation (Wu, Li, Cui, Eskin, & Goff, 2012; Zhang, Li, Wang, Zhang, & Peter, 2011). The biological activities of polysaccharides may be enhanced by means of chemical or physical modifications due to their wide spectrum of activities and potential in food industry, pharmaceuticals and biomaterials (Fox, Li, Xu, & Edgar, 2011). There has been growing interests in sulfated polysaccharides due to their better biological activities, such as anticoagulant, antiviral, antitumor and immunomodulation (Nishimura et al., 1998; Yi et al., 2012). Several researchers have shown the interest in regioselective synthesis of polysaccharides derivatives with different substitution positions, i.e. cellulose (Fox et al., 2011), curdlan (Zhang & Edgar, 2014), chitosan (Skorik, Gomes, Vasconcelos, Teresa, & Yatluk, 2003) and gellan exopolysaccharide (Redouan et al., 2011). Nishimura et al. (1998) also report the C-6 sulfated chitosan and C-2/C-3 sulfated derivatives exhibit distinct activities of anticoagulant and anti-AIDS, respectively. It is note-worthy that ionic groups in different substitution positions may have a variety of functions and biological activities.

Due to the structure diversities, the relationship between biological activity and solution conformation is complicated. Interestingly, water soluble polysaccharides with similar chemical structure exhibit different chain conformations in solutions, such as helix (triple, double and single), rigid rod, flexible chain, aggregate and sphere-like conformation (Zhang, Li, et al., 2011). Some researchers have concentrated on the relations between solution conformation and biological activity of natural or synthetic polysaccharides. Lentinan exists as triple helical structure in nature leading to greater antitumor activity than single helix (Tao, Zhang, & Peter, 2006). A water-soluble polysaccharide isolated from *Lentinus edodes* with triple helix chain presents significant antitumor bioactivities on Sarcoma180 solid tumor cell (Zhang, Gu, et al., 2010;

* Corresponding author at: College of Life Science, Northwest Normal University, Anning Eastroad, 967 Lanzhou, People's Republic of China. Fax: +86 0931 7971414.
*E-mail address:* nuno-vai@163.com (J. Wang).

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Zhang, Li, & Zhang, 2010). A conformational transition of branched (1→4)-α-D-glucan from *Rhizoma Panacis Japonici* is observed in its sulfated (S-RPS3) and phosphated (P-RPS3) derivatives. S-RPS3 and P-RPS3 significantly inhibit H-22 tumor cells growth with the transition of sphere-like conformation to random coil-like conformation (Chen et al., 2014). These results indicate that the expanded chain conformation is benefit to bioactivities of sulfated polysaccharides.

*Artemisia sphaerocephala* Krasch is widely distributed in Gansu province and Inner Mongolia Autonomous Region, China. In our earlier studies, *A. sphaerocephala* polysaccharide (ASP) is extracted and purified. ASP is an acidic polysaccharide with the uronic acid content of 16.3%. Monosaccharide composition analysis showed that ASP consists of arabinose, xylose, mannose, glucose and galactose (molar ratio of 1:4.2:45.9:9.7:11.4). The weight average molecular weight ($M_W$) and polydispersity (PD) of ASP was measured to be $7.348 \times 10^4$ Da and 3.184, respectively (Wang et al., 2015). Sulfated *Artemisia sphaerocephala* polysaccharide (SASP) was prepared using chlorosulfuric acid/pyridine method. SASP exhibited enhanced scavenging activities of radicals and reducing power, which was due to the high DS and more rigid conformation (Wang et al., 2014). Furthermore, regioselective synthesis of sulfated ASP ($S_{RS}$ASP) using triphenylchloromethane (TrCl) as protecting reagent was achieved. The DS of $S_{RS}$ASP varied from 0.44 to 0.63 with the substitution positions at C-2 and C-3. The chemical structure was characterized by FT-IR, XPS, $^{13}$C NMR, GC–MS (monosaccharide composition) and size-exclusion chromatograph combined with multi-angle laser photometer (SEC-MALLS) analysis (Wang et al., 2015).

The objective of this study is to investigate solution properties of $S_{RS}$ASP. SEC-MALLS, circular dichroism (CD), methylene blue (MB) and congo red (CR) spectrophotometric method were employed to study the conformation parameters and transition features. Atomic force microscopy (AFM) was used to observe the shape and size of the polysaccharide molecules. Antioxidant experiments of $S_{RS}$ASP with different substitution positions were employed to study the relationship between solution conformation and biological activity. The antioxidant activities were evaluated in vitro including scavenging activities against 1,1-diphenyl-2-picrylhydrazyl (DPPH), superoxide and hydroxyl radicals, chelating ability and reducing power.

## 2. Materials and methods

### 2.1. Materials

All chemicals such as ascorbic acid (Vc), butyl hydroxy anisole (BHA) and ethylene diamine tetraacetic acid (EDTA) were purchased from Jingchun Industry Co. Ltd. (Shanghai, China). The chemicals were of analytical reagent without further purification. The extraction and purification of ASP was reported in our previous studies (Wang et al., 2010).

### 2.2. Regioselective sulfation of ASP

The regioselective sulfation of ASP was reported in our previous study (Wang et al., 2015). In brief, ASP (500 mg) was dissolved in 30 mL anhydrous formamide and 5 mL pyridine. The polysaccharide solution was stirred for 30 min. Then, different amounts of TrCl (0.2–2.5 g) was added and stirred at 80 °C for 12 h. Then, ethanol (100 mL) was added to precipitate the products ($ASP_{Tr1-5}$).

Sulfated $ASP_{Tr}$ ($SASP_{Tr}$) was prepared according to our previous studies (Wang et al., 2014). Chlorosulfuric acid (CSA) was added dropwise in anhydrous pyridine filled in a three-necked flask, under agitating and cooling in ice water bath. $ASP_{Tr}$ (500 mg) with different triphenyl content was added to 20 mL anhydrous formamide at room temperature with stirred for 30 min. Then sulfating reagent was added. After 3 h, the mixture was neutralized (pH value was adjusted to 7–8 with 2 mol/L NaOH), precipitated, dialyzed (molecular weight cutoff 8–12 kDa) and lyophilized to give sulfated $ASP_{Tr}$ ($SASP_{Tr1}$–$SASP_{Tr5}$).

$SASP_{Tr}$ (500 mg) was added to dichloroacetic acid (15 mL) portionwise for dissolution to remove the Tr group. The solution was stirred at room temperature for 1 h. Then, ice water (100 mL) was poured slowly. The products were collected by filtration, washed with ethanol and lyophilized. Five deprotected $SASP_{Tr}$ ($S_{RS}ASP_1$–$S_{RS}ASP_5$) were kept in drybox before use.

Element Analysis (Euro Vector EA3000, Leeman) was employed to determine the sulfur contents. The degree of substitution ($DS_S$) was calculated according to the equation:

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

### 2.3. SEC-MALLS measurement

The weight average molecular weight ($M_W$) and mean square radius of gyration ($\langle S^2 \rangle_z$) of the samples were determined by employing size-exclusion chromatograph combined with multi-angle laser photometer (SEC-MALLS, λ = 690 nm; DAWN EOS, Wyatt Technology Co., USA). SEC-MALLS measurements were performed on multi-angle laser light scattering equipped with an Ultrahydrogel™ column (7.8 × 300 mm, Waters, USA). An optilab refractometer (Dawn, Wyatt Technology Co., USA) was also connected with this system. Samples were dissolved in ultrapure water with the concentration of 0.01 mg/mL and filtered through a cellulose filter. The mobile phase was ultrapure water at a flow rate of 0.5 mL/min (injection volume of 50 μL). The refractive index increment ($dn/dc$) value was determined to be 0.145 mL/g. The $M_W$ and $\langle S^2 \rangle_z$ were calculated by the Zimm method. The Astra software (Wyatt Tech. Corp.) was used for data analysis.

### 2.4. Methylene blue and congo red binding studies

Methylene blue (MB) spectrophotometric method was carried out according to the report of Antonov with some modifications (Antonov & Sato, 2009). MB was dissolved in distilled water by stirring at room temperature for 1 h. The MB concentration was adjusted to 0.0005 wt% with the absorbance of 0.7–0.8 at 664 nm (20 °C). Polysaccharide/MB complexes were prepared by dropwise addition of the solution of polysaccharide to a solution of MB, to give a final polysaccharide concentration of 0.005–0.5 mg/mL. Ultraviolet–visible absorption spectra were recorded in the range from 450 to 750 nm (UV1000, Labtech).

Congo red (CR) spectrophotometric method was performed according to the report of Rout, Mondal, Chakraborty, and Islam (2008). CR was dissolved in distilled water (91 μmol/L) by stirring at room temperature for 1 h. Then, 2 mL polysaccharide solution (0.2–2 mg/mL) was mixed with 1 mL NaOH and 2 mL CR solution, to give a final NaOH concentration of 0.2–2 mol/L. Meanwhile, mixed solution without polysaccharide was prepared as the control. After equilibrating for 10 min, the absorbance was measured in the range from 400 to 600 nm at room temperature (UV1000, Labtech).

### 2.5. Circular dichroism

Circular dichroism (CD) measurements were carried out in the range from 200 to 300 nm (20 nm/min) with a Jasco J-810 spectropolarimeter (Jasco Corporation, Tokyo, Japan, 25 °C). A flat faced quartz sample cell of 10 mm optical path length was used. All