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

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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.

Keywords: *Artemisia sphaerocephala*; sulfated polysaccharide; regioselective synthesis; solution conformation; antioxidant activity

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 (Zhang et al., 2011a; Wu, Li, Cui, Eskin & Goff, 2012). 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, Li, Xu & Edgar, 2011), curdlan (Zhang & Edgar, 2014), chitosan (Skorik et al., 2003) and gellan exopolysaccharide (Redouan et al., 2011). Nishimura et al also report the C-6 sulfated chitosan and C-2/C-3 sulfated derivatives exhibit distinct activities of anticoagulant and anti-AIDS, respectively (Nishimura et al., 1998). 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 et al., 2011a). 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 et al., 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 7.348×10^4 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 (0.2~2.5g) was added and stirred at 80 °C for 12h. 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}ASP₁~S_{RS}ASP₅) 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:

$$DS = \frac{1.62 \times S\%}{32 - 1.02 \times S\%} \quad (\text{Wang et al., 2011b}) \quad (1)$$

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, $\lambda = 690$ nm; DAWN EOS, Wyatt Technology Co., USA). SEC-MALLS measurements were performed on multi-angle laser light scattering equipped with an UltrahydrogelTM 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 μ L). 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 et al (Rout, Mondal, Chakraborty & Islam, 2008). CR was dissolved in distilled water (91µmol/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 observations were repeated at least 3 times with a 2 nm bandwidth and a time constant of 2 s.

2.6 Atomic force microscopic

Atomic force microscopic (AFM) image for sulfated polysaccharide was taken in tapping mode with a Nanoscope IIIa controller (Veeco Instruments, USA). Sample was dissolved in ultrapure water and diluted to the concentration of 1 µg/mL. Two microlitres of the solution were pipetted onto a freshly cleaved mica and air dried.

2.7 Determination of antioxidant activities in vitro

The DPPH and superoxide free radical scavenging activities of the sulfated samples were investigated according to our previous studies (Wang et al., 2014). The hydroxyl radical scavenging activity was determined according to the report of Melo (Melo et al., 2013). The ferrous ion chelating ability of the samples was measured according to the report of Li (Li et al., 2013). The reducing power was measured according to the report of Qi et al (Qi et al., 2006).

All results were expressed as means±S.D. (n=3). EC₅₀ value was defined as the concentration that scavenged a 50% radical. Data and EC₅₀ values were analyzed by SPSS software 18.0.

3. Results and discussion

3.1 Solution conformation analysis

Multi-angle light scattering technique is the most common method for conformation analysis (Yi et al., 2012). Solution conformation can be calculated according to equation (2), where $\langle S^2 \rangle_z^{1/2}$ is mean square radius of gyration; M is

weight average molecular weight; f is equation coefficient (Tao, Zhang & Peter, 2006). The d_f value is defined as the inverse of the exponent ν (1 for rigid rod chain, 0.5 for Gaussian coil and 0.3 for three-dimensional globular shape):

$$\langle S^2 \rangle_z^{1/2} = fM^\nu \quad (2)$$

$$d_f = 1/\nu \quad (3)$$

In this study, we investigated the effects of molecular weight, DS and substitution positions on the solution conformation of sulfated polysaccharides using regioselective synthesis. The values of DS, M_w , d_f and straight line fitting of the experimental data points were summarized in Table 1. Fig. 1 showed the log-log plot of M_w versus $\langle S^2 \rangle_z^{1/2}$ of the samples. The d_f value of 2.86 showed that ASP existed as a conformation between the hard sphere ($d_f=3.0$) and the fully swollen branched macromolecule ($d_f=2.0$) in aqueous solution. Compared to ASP, a decrease in M_w was observed in all sulfated samples. The result indicated that an extensive degradation of $S_{RS}ASP$ occurred during the reaction (Table 1). Moreover, a decrease in d_f value in all sulfated samples was observed. The d_f values of $S_{RS}ASP_1$ - $S_{RS}ASP_4$ were varied from 1.56 to 1.85. Normally, the polymer adopts a random coil conformation when d_f value between 1.5 to 2.0. It indicated that $S_{RS}ASP_{1-4}$ exhibited as a more rigid conformation of random coil in aqueous solution. $S_{RS}ASP_5$ showed relatively high d_f value of 2.04, which lie in the range of hard sphere and the branched polymers (not swollen). The d_f value between 2-2.5 could be due to the steric hindrances of macromolecules or thermodynamic reasons (Bauer, & Burchard, 1993).

Table 1 Molecular weight and d_f values of sulfated derivatives

Samples	DS	$M_w \times 10^4$ PD ^a	Relation equation	d_f	Conformation
ASP	- ^b	7.348	$\langle S^2 \rangle_z^{1/2} = 0.203M_w^{0.35 \pm 0.027}$	2.86	between hard sphere and random
		3.18			coil (fully swollen)
SASP	0.93	3.203	$\langle S^2 \rangle_z^{1/2} = 0.141M_w^{0.42 \pm 0.044}$	2.38	between hard sphere and random
		2.02			coil (not swollen) ^c
S _{RS} ASP ₁	0.61	1.867	$\langle S^2 \rangle_z^{1/2} = -0.950M_w^{0.62 \pm 0.088}$	1.61	random coil
		2.17			
S _{RS} ASP ₂	0.57	1.806	$\langle S^2 \rangle_z^{1/2} = 0.201M_w^{0.59 \pm 0.051}$	1.69	random coil
		2.22			
S _{RS} ASP ₃	0.63	3.272	$\langle S^2 \rangle_z^{1/2} = 0.150M_w^{0.54 \pm 0.072}$	1.85	random coil
		3.87			
S _{RS} ASP ₄	0.58	3.203	$\langle S^2 \rangle_z^{1/2} = 0.167M_w^{0.64 \pm 0.067}$	1.56	random coil
		3.55			
S _{RS} ASP ₅	0.44	3.255	$\langle S^2 \rangle_z^{1/2} = 0.361M_w^{0.49 \pm 0.066}$	2.04	between hard sphere and random
		2.85			coil (not swollen)
SASP _{cata2}	1.24	3.103	$\langle S^2 \rangle_z^{1/2} = -1.99M_w^{0.88 \pm 0.55}$	1.13	between rigid rod and random coil ^d
		1.28			

^a Data from our previous report (Wang et al., 2015)^b Not detected^c Data from our previous report (Wang et al., 2010)

^d Data from our previous report (Wang et al., 2014)

Some researchers have concentrated on the relations between molecular weight and solution conformation of polysaccharides. Zhang et al studied the effect of molecular weight on solution conformation of lentinan. Triple helical lentinan with high M_w (1.71×10^6 Da) formed a self-entangle conformation, no ordered aggregates were observed. Conversely, short triple helical chains (5×10^5 Da) aligned to each other and formed “faggot-like” clusters. Molecular weight had great effect on solution conformation. Rigid conformation such as the rod-like chains with low molecular weight could form ordered aggregates as a result of the strongest interaction between each chain (Wang, Xu & Zhang, 2008; Zhang, Li & Zhang, 2010). The conformation change of fragmented *ι*-carrageenan with shorter chain was reported by Rees et al. Low molecular weight *ι*-carrageenan exhibited a disorder-order transition without the gelation that was a feature of the behavior of the natural polymer (Rees, Williamson, Frangou & Morris, 1982). Chen et al also reported that RPS3 exhibited condensed coil conformation. The sulfated and phosphorylated RPS3 showed more extended coil conformation (Chen et al., 2014). The result indicated that the chain extensibility depended on the molecular weight, type of substituent groups and DS. This result was in accordance with our earlier report that lower M_w might lead to a rigid conformation (Wang et al., 2010; Wang et al., 2014).

Fig. 1 Log-log plot of M_w versus $\langle S^2 \rangle_z^{1/2}$ of ASP, SASP and $S_{RS}ASP$ at 25°C

For a better understanding the conformational changes, we also compared the d_f values of sulfated polysaccharides using different synthetic strategies. As already shown in our previous studies, sulfated ASP ($SASP_{cata2}$, using catalytic synthesis) with high DS of 1.24 showed a solution conformation between rigid rod and random coil (Wang et al., 2014). SASP (using conventional CSA/pyridine method) with a DS of 0.93 exhibited an internal structure between hard sphere and random coil (not swollen). Meanwhile, $S_{RS}ASP_5$ with lower DS of 0.44 showed higher d_f value compared to $S_{RS}ASP_1$ - $S_{RS}ASP_4$. From these results, it could be concluded that the solution conformation was strongly dependent on DS. Similar result was reported by Tao et al that a d_f value of 1.79 was obtained in sulfated polysaccharide. It might be

due to the $-\text{SO}_3\text{H}$ groups enhance the steric hindrance between the biomacromolecule chains (Tao, Zhang & Peter, 2006). Chen et al also reported that the sulfated and phosphorylated derivatives exhibited relatively high values of ν (Chen et al., 2014).

However, SASP with high DS showed a d_f value of 2.38, which was higher than that of $\text{S}_{\text{RS}}\text{ASP}$ (d_f value of 1.56-2.04) with similar M_w but lower DS. It could be attributed to the fact that, in regioselective sulfation, electrostatic repulsive interactions caused by different substitution positions were not be neglected. The solution conformation was mainly governed by the dual inter-play between intramolecular hydrogen bonding and elastic contributions of polymer chains (i.e. molecular weight and electrostatic interactions of polymer chains) (Wang et al., 2014). Sulfated polysaccharides showed a significant expanded conformation upon increasing of $-\text{SO}_3\text{H}$ groups, which exhibited different intramolecular hydrogen bonding compared to nonionic polysaccharides (Fig. 2). Similar results were also reported that $-\text{SO}_3\text{H}$ groups caused the increasing of stiffness and expansion due to the electrostatic repulsion of polymer chains (Chen, Xu, Zhang & Zeng, 2009). Compared with the samples substituted at C-6, the decrease in d_f value was attributed to the breakup of rigid hydrogen bonds from secondary hydroxyl groups in C-2 and C-3 substituted polysaccharide, which evidenced a more expanded conformation in aqueous solution (Fig. 2). It was shown that the substitution position of $-\text{SO}_3\text{H}$ group was an important factor to influence the solution conformation of sulfated polysaccharides.

Fig. 2 Schematic representation of solution conformation transition after regioelective sulfation

3.2 Circular dichroism (CD) analysis

CD is employed as a convenient method for the characterization of conformational changes of macromolecules with optical activity, which may affect the polarizability and orientation of the chromophores. The CD spectra were shown in Fig. 3 in which $SASP_{cata2}$ and $S_{RS}ASP_4$ was compared with ASP at neutral pH value. The CD spectrum of ASP exhibited a negative peak maximum at 209 nm, which might attribute to the $n-\pi^*$ transition of carboxylate. It was in accordance with our earlier report that ASP is an acidic polysaccharide (Wang et al., 2010).

Fig. 3 Circular dichroism spectra of ASP and its sulfated derivatives at 25°C

Like ASP, $S_{RS}ASP_4$ exhibited negative ellipticity in the concentration of 0.8 and 1.0 mg/mL (Fig. 3). However, in comparison with ASP, the maximum was decreased and red-shifted to 219 nm. Our previous report also showed that a decrease of the ellipticity and red shift of $n-\pi^*$ transition at 216 nm was observed in $SASP_{cata2}$ (Wang et al., 2014). The signal intensity was approximately 6 times less for $SASP_{cata2}$ and $S_{RS}ASP_4$ compared with ASP. The new ellipticity and shift to a lower energy excitation wavelength near 220 nm could be attributed to chromophore contributions of the introduced $-SO_3H$ group of sulfated polysaccharides (Forget et al., 2013). CD scans agreed well with the SEC-MALLS result that the red shift of $n-\pi^*$ transition and the decrease in ellipticity indicated the appearance of $S=O$. It therefore appeared that

the sulfation of the polysaccharide promoted a transition of the chains leading to an expanded conformation.

3.3 Polysaccharide-methylene blue interactions

The studies of the binding of dyes to polymers have become a common technique to identify molecular conformation of water soluble macromolecules, such as polysaccharides, nucleic acids and proteins (Antonov & Sato, 2009). Methylene blue (MB), a planar cationic phenothiazinium dye, to form complex in the presence of an anionic polysaccharide results in changes in the absorption spectrum of the dye (Girod et al., 2004). MB displayed a characteristic absorption band centered at 664 nm together with a shoulder at 619 nm, which was attributed to the monomeric and dimeric dye, respectively (Michon, Konate, Cuvelier & Launay, 2002). The evolution of the absorption values determined at 664 nm (A_{664}) and 554 nm (A_{554}) as a function of sample concentrations were investigated. Fig. 4A showed the absorption spectra of $S_{RS}ASP_4$ -MB complexes which contains various amounts of $S_{RS}ASP_4$ (0.005-0.5mg/mL) in a definite concentration of MB. As shown in Fig. 4A, remarkable hypochromic effect at 664 nm and hyperchromic effect at 554 nm were observed especially for the sample with higher polysaccharide concentrations. A_{664} decreased and A_{554} increased as $S_{RS}ASP_4$ concentration increased up to a limit of 0.05 mg/mL, indicating that there was an equilibrium state between sulfated polysaccharide and MB (Fig. 4B). It could be assumed that all the positively charged MB molecules were saturated by sulfated groups. Girod et al also reported a similar

result of an ‘optimal’ concentration in *ι*-carrageenan/MB interactions (Girod et al., 2004).

It was reported that MB and sulfated polysaccharides could form metachromatic complexes in which MB molecules in parallel absorption near 560 nm. Long-range forces involving MB and H₂O could take place and maintain the planar MB molecules relatively close to each other (Michon, Konate, Cuvelier & Launay, 2002). This equilibrium could be reversed on heating and the spectrum of the complex then approached to that of the MB alone. It indicated that the MB molecules bound to the anionic polymer in the rigid conformation (i.e. double-helix), but not in the flexible form (Rees, Williamson, Frangou & Morris, 1982; Zhang et al., 2011a). The dissociation of a duplex ct-DNA into two single strands induced by heating was also reported by Mudasir et al in nucleic acid-MB binding test. The denaturation temperature was depended on the strength and mode of nucleic acid-MB interactions (Mudasir et al., 2010). In this case, we investigated the effect of temperature on the conformational transition. The evolutions of A_{664} and A_{554} as a function of temperature (20-80°C) were shown in Fig. 4C and 4D. The degree of hypochromicity at 554 nm increased with the increasing of temperature. As can be seen from Fig. 4D, the hypochromic effect of A_{554} decreased from 0.231 to 0.128 by increasing temperature from 40°C to 80°C. On the contrary, the hyperchromic effect of MB solution in the presence of S_{RS}ASP₄ was found to increase from 0.329 to 0.896 when the temperature increased from 40°C to 80°C. This was in accordance with our earlier result that the transition temperature of solution conformation was near 50°C in polysaccharide-MB

complexes (Wang et al., 2014). The remarkable hypochromic effect at 554 nm indicated the existence of strong interaction between sulfated polysaccharide and MB molecules, most probably via electrostatic attraction. The interaction of binding resulted in ordered stacking of MB and stabilized electronically in the helical conformation of sulfated polysaccharide. Compared to SASP_{cata2} with higher DS and more rigid conformation, S_{RS}ASP₄ showed greater hyperchromic and hypochromic effects at 664 nm and 554 nm, respectively. It might be due to the lower DS and more flexible conformation of S_{RS}ASP₄. However, S_{RS}ASP₄ with higher d_f value of 1.56 showed less hypochromic effect at 664 nm compared to SASP with higher DS but lower d_f value of 2.38 (Wang et al., 2014). Rees et al reported that the driving forces toward binding on the polymer from a dye-stacking tendency which might involve the electrostatic interactions due to the rigidity of the solution conformation (Rees, Williamson, Frangou & Morris, 1982). It could be concluded that DS and d_f values are key factors to influence the absorption spectra of dye-polysaccharide complexes.

Fig. 4 Dye-polysaccharide binding tests (A) Absorption spectra of MB measured from 450 nm to 750 nm at different concentrations of $S_{RS}ASP_4$ at 20°C. (B) Evolution of absorption values at 664 nm and 554 nm for $S_{RS}ASP_4$ as a function of polysaccharide concentrations. (C) Absorption spectra of MB at different temperatures with $S_{RS}ASP_4$ concentration of 0.05 mg/mL. (D) Evolution of absorption values at 664 nm and 554 nm for $S_{RS}ASP_4$ as a function of temperatures. (E) Evolution of absorption values of $S_{RS}ASP_4$ -CR as a function of polysaccharide concentrations at 20°C. (F) Evolution of absorption values of $S_{RS}ASP_4$ -CR as a function of NaOH concentrations at 20°C.

3.4 Polysaccharide-congo red interactions

The conformational transition of the sulfated polysaccharides was determined by employing CR-polysaccharide complexes spectrophotometric method at various alkali concentrations. As shown in Fig. 4E, compared to the curve of SASP_{cata2} obtained in our earlier report, the maximum in the visible absorption (λ_{max}) of S_{RS}ASP₄-CR complexes red-shifted to longer wavelengths at the polysaccharide concentrations range of 0-2.0 mg/mL. It was indicated that S_{RS}ASP₄ adopted a highly ordered conformation in CR-polysaccharide complex. Then, the complex formation of S_{RS}ASP₄-CR was determined from the red-shift in alkaline conditions at the polysaccharide concentration of 1 mg/mL. The λ_{max} of S_{RS}ASP₄-CR decreased substantially at the NaOH concentrations range of 0-2.0 M, which indicated that the highly ordered conformation of S_{RS}ASP₄ might convert into a flexible conformation (Fig. 4F).

Generally, at the NaOH concentration below 0.1 M, the λ_{max} red-shifts to longer wavelength (beyond 505 nm) which is attribute to a triple-helical structure of polysaccharide (Zhao et al., 2014). The λ_{max} of CR were largely red-shifted (from 500 to 514 nm) by the presence of *Pleurotus florida* polysaccharide (PFPSIN) in 0.1 M NaOH solution. It suggested that PFPSIN had triple helical structure (Rout, Mondal, Chakraborty & Islam, 2008). Zhang et al also showed that *Lentinus edodes* polysaccharide existed as a triple-helix in 0.1M NaOH (Zhang et al., 2010). Our result showed the order-disorder transition of the CR-polysaccharide complexes due to the

breakage of intermolecular hydrogen bonds in alkaline solution. Thus, it could be concluded that $S_{RS}ASP_4$ showed a rigid conformation as evidenced in SEC-MALLS. However, no triple-helical conformation was detected. Similar result was reported that longan pulp polysaccharide showed a transition from rigid conformation to flexible conformation due to the depolymerization of polysaccharide-dye complex (Yi et al., 2012).

3.5 AFM

AFM has been widely employed to investigate the molecular and supramolecular structures of macromolecules, especially for the samples with soft biological surface (Yi et al., 2012). ASP exhibited as a linear molecule or network structure at the concentrations of 1 $\mu\text{g/mL}$ in AFM image (Zhang et al., 2007). Fig. 5 showed the AFM images of $S_{RS}ASP_4$. In order to avoid the influence of tip broadening effects, molecular height in section analysis was used to characterize the size of particles. The images revealed that $S_{RS}ASP_4$ was mainly spherical lumps and rod-like morphologies. The particles were inhomogeneous with the mean height of 2.406 ± 0.79 nm and mean diameter of 95.7 ± 17.9 nm in particle analysis. The width and height of the rod-like structures ranged from 70 to 160 nm and 1.2 to 1.8 nm in section analysis, respectively. Generally, the height of a single polysaccharide chain is about 0.1-1 nm. It suggested that intermolecular and intramolecular aggregation might be involved in $S_{RS}ASP_4$. It was ascribed to the hydroxyl and $-\text{SO}_3^-$ groups on the chains of the $S_{RS}ASP_4$ providing the strong intermolecular and intramolecular interaction. However,

linear molecules and tangled network structures were not observed in $S_{RS}ASP_4$. Based on the result, we could conclude that there was remarkable effect on the molecule conformation for the polysaccharides after sulfation.

Fig. 5 2D/3D AFM images and section analysis of $S_{RS}ASP_4$ at the concentration of 1 µg/mL

3.6 Evaluation of antioxidant activities *in vitro*

It was reported that the functional groups such as $-SO_3^-$, $-OH$ and $-COOH$ were responsible for the biological activities of polysaccharides. The antioxidant activities of sulfated polysaccharides were related to many factors, such as substitution groups, average molecular weight, DS and substitution positions (Melo et al., 2013). In order

to study the effect of regioselective synthesis on the bioactivity of sulfated polysaccharides, antioxidant experiments of sulfated ASP with different substitution positions were carried out. $S_{RS}ASP_4$ and $SASP_{cata2}$ were selected to evaluate the antioxidant activities for their similar M_w (3.203 and 3.103×10^4 Da, respectively) but different DS and substitution positions. The data of $SASP_{cata2}$ was reported in our previous study (Wang et al., 2014). Methods in the different stages including reducing power (initiation), chelation of iron ion (propagation) and scavenging of DPPH, superoxide and hydroxyl radicals (termination) were employed to evaluate the antioxidant activities in vitro. EC_{50} value was defined as the concentration that scavenged a 50% radical.

3.6.1 DPPH radicals scavenging activity

The scavenging abilities of sulfated ASP on DPPH radical were shown in Fig. 6A and compared with Vc and ASP. The result indicated that all samples showed dose-dependent features in the concentration ranges of 0.02-5 mg/mL. Both $SASP_{cata2}$ and $S_{RS}ASP_4$ had abilities on scavenging DPPH radicals, the EC_{50} values were 2.007 and 3.988 mg/mL, respectively. Sulfated ASP showed higher scavenging abilities than ASP (EC_{50} of 4.946 mg/mL) but lower than Vc (EC_{50} of 0.631 mg/mL). $SASP_{cata2}$ exhibited lower EC_{50} value than $S_{RS}ASP_4$ which indicated that high DS could enhance the scavenging abilities of DPPH radicals. Redouan et al reported that higher uronic acids content and sulfate groups could enhance the antioxidant activity of gellan exopolysaccharide (Redouan et al., 2011). Other authors also reported that

modification of xanthouronan by regioselective oxidation could considerably enhance the anti-DPPH radical properties (Delattre et al., 2015). The addition of anionic groups at C-2 and C-3 such as carboxylate and sulfate could activate the hydrogen atom of the anomeric carbon and reduce the intramolecular/intermolecular hydrogen bond, thus promoting hydrogen-donating abilities (Melo et al., 2013). It was in accordance with our earlier result that SASP_{cata2} with highest DS exhibited the best scavenging DPPH radical activity (Wang et al., 2014).

3.6.2 Superoxide radical scavenging activity

The superoxide radicals scavenging abilities of samples showed significance in a concentration-dependent fashion (Fig. 6B). The scavenging effect of the samples increased in the order of ASP, S_{RS}ASP₄ and SASP_{cata2} with the EC₅₀ values of 1.013, 0.131 and 0.027 mg/mL, respectively. Similar result of sulfated polysaccharides with the EC₅₀ values of 0.9-0.02 mg/mL was reported. Carboxylic groups with strong electron-withstanding ability in the modified polysaccharides enhanced the activity of scavenging superoxide radical (Redouan et al., 2011). Qi et al also reported that the acetylated and benzoylated ulvans could terminate radical chain reactions by stabilization of free radicals in carbohydrate chains and exhibit more pronounced superoxide radical scavenging abilities (Qi et al., 2006). Zhang et al reported that sulfated *Auricularia auricular* polysaccharides showed stronger scavenging activity on the superoxide radical than Vc at the concentration of 2.0 mg/mL (Zhang et al., 2011b). These results concluded that anionic polysaccharides showed greater

antioxidant activity which is related to the contents of the substitution groups.

Fig. 6 Antioxidant effect of ASP and its sulfated derivatives. Samples with different substitution positions were selected. (A) scavenging activity of DPPH radicals; (B) scavenging activity of superoxide radicals; (C) reducing power evaluation; (D) scavenging activity of hydroxyl radicals; (E) ferrous ions chelating effect; (F) Schematic representation of the formation of chelates; data are presented as mean values (n=3).

3.6.3 Reducing power

The reducing abilities of the samples were shown in Fig. 6C. Upon comparing with BHA, the lower reducing agent potency of the samples could be observed. At all the concentrations, SASP_{cata2} and S_{RS}ASP₄ were more potent than ASP. The reducing power of ASP, S_{RS}ASP₄ and SASP_{cata2} was 0.052, 0.059 and 0.102 at the concentration of 1 mg/mL, respectively. The reducing power of the polysaccharides with different structure features had been shown to exert antioxidant action by interrupting the free radical chain by donating a hydrogen atom (Li et al., 2013). Melo and colleagues proved that sulfated polysaccharides from *Dictyopteris justii* presented great reducing power. Polyanion, a good electron donor, with higher density of negative charges showed better reducing power (Melo et al., 2013).

3.6.4 Hydroxyl radical scavenging activity and chelating effect on ferrous ions

The hydroxyl radical is very active in reactive oxygen species (ROS), making it very harmful to bio-molecules in organism such as proteins, DNA and lipids. Hydroxyl radical can be generated by the reaction between transition metals and the superoxide ion in the Fenton reaction. Two types of antioxidant mechanism were reported: scavenging of hydroxyl radical and suppression against hydroxyl radical generation (Li et al., 2013; Melo et al., 2013; Zhang et al., 2011b). Fig. 6D described the scavenging activity on hydroxyl radical of the samples. Compared to ASP (EC₅₀ of 6.073 mg/mL), S_{RS}ASP₄ and SASP_{cata2} showed improved scavenging performances

with the EC₅₀ values of 5.03 and 2.14 mg/mL, respectively.

The second mechanism is more related to the transition of metal ions to specific metal complexes, which may prevent metal ions from interacting with H₂O₂, thus preventing the generation of hydroxyl radicals. In this case, the chelating ability of metal ions such as ferrous ions may affect the antioxidant activity. Fig. 6E showed a concentration-dependent manner of the ferrous ions chelating ability in all of the samples. At the concentration of 0.02-0.2 mg/mL, the chelating abilities of sulfated samples were weaker than ASP. At the higher concentration ranges of 0.6-5 mg/mL, S_{RS}ASP₄ showed the strongest chelating ability of 4.4-26.5%. Qi et al reported the great chelating ability ranged from 37% to 78% of acetylated ulvan at the concentration of 0.31-1.88 mg/mL (Qi et al., 2006). Similar result was reported that a moderate chelating ability of 23.7% and 27.6% was achieved in the sulfated polysaccharides extracted from *Dictyopteris justii*. Although many articles correlated the enhanced chelating ability to the presence of substituent groups, the sulfated glucans exhibited no ferric chelating activity (Melo et al., 2013). It was possible that the antioxidant ability was not a function of a one factor but a multiple action of many factors, such as DS, *M_w* and substitution patterns. Melo et al also noticed that both of sulfur content and the distribution of sulfate groups could influence the antioxidant activity (Melo et al., 2013).

Monosaccharides are the building blocks of polysaccharide which might have a great influence on chelating ability. In ASP, mannose, glucose and galactose are the main compositions of the polysaccharide chain (Wang et al., 2015). As we know,

neighbouring *cis*-hydroxyl groups possess higher coordination activity. Mannose residue in polysaccharide chain with *cis*-2,3-dihydroxy is beneficial to form Fe^{2+} -polysaccharide chelate in chelating experiment (Fig. 6F). In our previous studies, mannose was the dominant component in ASP. The ratio of mannose to glucose was 4.71 (45.9/9.74). However, glucose residue was the dominant monosaccharide in all sulfated samples. The ratio of mannose to glucose was 0.848 (12.41/14.62) for $\text{S}_{\text{RS}}\text{ASP}_4$ (Wang et al., 2015). This could explain the result observed in Fig. 6E that ASP showed higher chelating ability than $\text{S}_{\text{RS}}\text{ASP}_4$ and $\text{SASP}_{\text{cata}2}$ at low concentration. Moreover, the effect of substitution positions on the chelating activity should not be neglected. $\text{S}_{\text{RS}}\text{ASP}_4$ with the $-\text{SO}_3^-$ groups substituted at C-2 and C-3 might exhibit distinct chelating ability. Sulfate group at C-2 or C-3 was favorable for the formation of chelation with hydroxyl at ortho position, such as C-2($-\text{SO}_3^-$)/C-3($-\text{OH}$) and C-3($-\text{SO}_3^-$)/C-4($-\text{OH}$) chelates. Contrarily, sulfate group at C-6 might show weaker Fe^{2+} binding capacity due to the feature of exocyclic substituent group (Fig. 6F). The results indicated that the coordinated active sites were mainly supplied with sulfate group at C-2/C-3 and neighbouring *cis*-OH of main chain. It could be concluded that monosaccharide composition and substitution groups in a favorable structure-function configuration could show greater metal chelating activity in sulfated polysaccharides. Thus, further investigation is necessary to clarify this point.

4. Conclusion

In this study, a decrease in d_f values (1.56-2.04) of all sulfated samples was

observed. It attributed to the breakup of rigid hydrogen bonds from secondary hydroxyl groups in C-2 and C-3 substituted polysaccharides, which evidenced a more expanded conformation in aqueous solution. The red shift of $n-\pi^*$ transition and the decrease in ellipticity indicated a transition of the chains leading to an expanded conformation in CD analysis. S_{RS}ASP showed greater hyperchromic effect at 664 nm attributed to the low DS and more flexible conformation in MB binding experiments. It was indicated that DS and d_f values are key factors to influence the absorption spectra of dye-polysaccharide complexes.

Moreover, antioxidant evaluation revealed that S_{RS}ASP₄ could enhance the DPPH, superoxide and hydroxyl radicals scavenging activities and chelating abilities in vitro. In summary, our results indicated that the combined action of substitution position and DS may influence the biological activities of modified polysaccharides. Further studies about the antitumor activities of sulfated ASP with different substitution positions are in progress.

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Figure Captions:

Fig. 1 Log-log plot of M_w versus $\langle S^2 \rangle_z^{1/2}$ of ASP, SASP and S_{RS}ASP at 25°C

Fig. 2 Schematic representation of solution conformation transition after regioselective sulfation

Fig. 3 Circular dichroism spectra of ASP and its sulfated derivatives at 25°C

Fig. 4 Dye-polysaccharide binding tests (A) Absorption spectra of MB measured from 450 nm to 750 nm at different concentrations of S_{RS}ASP₄ at 20°C (B) Evolution of absorption values at 664 nm and 554 nm for S_{RS}ASP₄ as a function of polysaccharide concentrations. (C) Absorption spectra of MB at different temperatures with S_{RS}ASP₄ concentration of 0.05 mg/mL. (D) Evolution of absorption values at 664 nm and 554 nm for S_{RS}ASP₄ as a function of temperatures. (E) Evolution of absorption values of S_{RS}ASP₄-CR as a function of polysaccharide concentrations at 20°C (F) Evolution of absorption values of S_{RS}ASP₄-CR as a function of NaOH concentrations at 20°C

Fig. 5 2D/3D AFM images and section analysis of S_{RS}ASP₄ at the concentration of 1 µg/mL

Fig. 6 Antioxidant effect of ASP and its sulfated derivatives. Samples with different substitution positions were selected. (A) scavenging activity of DPPH radicals; (B) scavenging activity of superoxide radicals; (C) reducing power evaluation; (D) scavenging activity of hydroxyl radicals; (E) ferrous ions chelating effect; (F) Schematic representation of the formation of chelates; data are presented as mean values (n=3).