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# Sulfated modification, characterization and structure–antioxidant relationships of *Artemisia sphaerocephala* polysaccharides

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ABSTRACT

Sulfated polysaccharides exerted potent biological property which was relative to degree of sulfation (DS), $M_w$, substitution position and chain conformation. In the present study, sulfated derivatives of *Artemisia sphaerocephala* polysaccharide (ASP) with different DS were synthesized by chlorosulfuric acid/pyridine method. FT-IR and $^{13}C$ NMR analysis indicated that C-6 substitution was predominant in sulfated ASP (SASP) compared with other positions at C-2. In the sulfation reaction, a sharp decrease in $M_w$ was observed in SASP. The $d_f$ values from 1.96 to 2.77 indicated that the $-SO_3H$ groups leading to the relatively expanded conformation of SASP. Antioxidant assays showed that SASP had better antioxidant activities. The data obtained in vitro models indicated that high DS and moderate $M_w$ showed the best antioxidant capacities.



## 1. Introduction

Plant polysaccharides have been widely studied for their chemical properties and biological activities in food and medical industry (Wu, Cui, Tang, & Gu, 2007). Biological activities of a polysaccharide depend on its molecular structure including sugar unit and glycosidic bond of the main chain, the types and polymerization degree of the branch and flexibility and apetial configuration of the chains (Lu, Wang, Hu, Huang, & Wang, 2008). Therefore, molecular modification and structure improvement of polysaccharide arouse wide concern. Most studies have demonstrated that biological activities of polysaccharide are greatly increased by molecular modification (Xing et al., 2005). Recently, chemical modifications of polysaccharides by esterification, oxidation and hydroxypropylation are generally done for preparing custom-made derivatives having desirable functionality attributes (Parvathy, Susheelamma, Tharanathan, & Gaonkar, 2005). Sulfated polysaccharides comprise a complex group of macromolecules with a wide range of important biological properties. The sulfation of polysaccharides could not only enhance the water solubility but also change the chain conformation, resulting in the alteration of their biological activities (Liu et al., 2009). Many studies have confirmed that the sulfated polysaccharides exerted potent biological properties in comparison with non-sulfated polysaccharides, such as anti-coagulant, anti-virus, antioxidant and antitumor activities (Wang, Li, & Chen, 2009).

Oxidation is essential to many organisms for the production of energy to fuel biological processed (Xu et al., 2009). Oxidative stress, induced by oxygen radicals, is believed to be a primary factor in various diseases such as cancer, rheumatoid arthritis and atherosclerosis as well as in degenerative processes of aging. Reactive oxygen species (ROS), in the forms of superoxide anion ($^{\bullet}O_2^-$), hydroxyl radical ($^{\bullet}OH$) and hydrogen peroxide ($H_2O_2$), are generated by normal metabolic processes or from exogenous factors and agents, and they can easily initiate the peroxidation of membrane lipids, leading to the accumulation of lipid peroxides (Finkel & Holbrook, 2000). ROS can cause damage to a wide range of essential bio-molecules, such as DNA, and they have been associated with carcinogenesis, coronary heart disease and many other health problems related to advancing age (Zou et al., 2008).

Polysaccharides have exhibited strong antioxidant properties and can be explored as novel potential antioxidants (Tseng, Yang, & Mau, 2008). It was reported that the molecular weight of polysaccharides was an important parameter influencing antioxidant activities. The polysaccharide fraction (TPC-3) isolated from *Camellia sinensis* with lowest molecular weight ($4.2 \times 10^4$ Da) showed the highest antioxidant activities (Chen, Zhang, Qu, & Xie, 2008; Chen, Xie, Nie, Li, & Wang, 2008). One sulfated lacquer polysaccharide,

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with moderate $M_w$ ($1.27 \times 10^4$ Da) showed the best antioxidant capacities. Its reducing capacity was 0.61 at 500 μg/mL, scavenging ability for superoxide and hydroxyl radical were 56.4% at 500 μg/mL and 55.6% at 1000 μg/mL, respectively (Zou et al., 2008). Although the author was not mentioned, sulfated fungus polysaccharide (YSP-S2) with low molecular weight ($0.45 \times 10^6$ Da) showed the best antioxidant capacities (Yang, Du, Huang, Wan, & Wen, 2005; Yang, Gao, Han, & Tan, 2005).

The genus Artemisia belongs to the family Compositae (Asteraceae) and has been used in folk medicine to treat a variety of diseases, such as hepatitis, fever and malaria, rheumatoid arthritis and asthma (Xie et al., 2008). *Artemisia sphaerocephala* Krasch is widely distributed in Gansu province and Inner Mongolia Autonomous Region, China. It is widely used in food industry as food thickener and stabilizer (Chinese flora editorial committee, 1991). In our previous studies, we have extracted *A. sphaerocephala* polysaccharides (ASP) by hot water extraction. The composition of ASP is L-Ara, D-Xyl, D-Lyx, D-Man, D-Glu and D-Gal (molar ratio of 1:4.64:1.74:27.6:3.90:14.4) with the molecular weight ($M_w$) of $1.42 \times 10^5$ Da (Zhang et al., 2007). At a dose of 200 mg/kg body weight, ASP produced a significant decrease in blood glucose, plasma cholesterol and plasma triglycerides levels in alloxan-induced diabetic rats, indicating that ASP have hypoglycemic activity (Zhang, Huang, Hou, & Wang, 2006).

In the present study, sulfated derivatives of ASP (SASP) with different degrees of substitution (DS) were prepared with chlorosulfuric acid (CSA)/pyridine in formamide. The structure of SASP was analyzed by FT-IR and $^{13}C$ NMR spectroscopy. Size-exclusion chromatograph combined with multi-angle laser photometer (SEC-LLS) was employed to determine the molecular weight, molecular weight distribution and chain conformation. The antioxidant properties of both ASP and its sulfated derivatives were evaluated and compared for the purpose of assessing the relationship between structure and bioactivity. Antioxidant properties were assayed in terms of antioxidant activities in vitro, by scavenging abilities on superoxide radicals, hydroxyl radicals, 1,1-diphenyl-2-picrylhydrazyl (DPPH), reducing power and chelating ability on ferrous ions.

## 2. Materials and methods

### 2.1. Extraction and purification of crude ASP

Microwave-assistance extraction (MAE) was performed on microwave apparatus using vessel system (NJC 03-2 Microwave experiment equipment, Nanjing, China). *A. sphaerocephala* seed power was put into a 3000 mL PTFE extraction vessel. The extraction condition was ratio of water to raw material 300:1, microwave power 500 W, extraction temperature 60 °C and extraction time 70 min. After extraction, the vessel was allowed to cool at room temperature, filtered and freeze-dried to obtain crude ASP.

The protein of crude ASP was removed by Sevage method joined papain according to the earlier report in our laboratory (Zhang et al., 2006). The purified ASP was obtained by gel permeation chromatography (GPC) on an Ultrahydrogel 500 column (Waters, USA), at a concentration of 1.000 g/L and flow rate of 0.08 mL/min. The purity of ASP collections was monitored under ultraviolet (UV) light at 280 nm.

### 2.2. Sulfated modification of ASP

#### 2.2.1. Preparation of sulfating reagent

Chlorosulfuric acid (CSA) was dropped one by one in anhydrous pyridine filled in three-necked flask, under agitating and cooling in ice water bath (Wang, Li, et al., 2009). All determinations were completed in 40 min.

#### 2.2.2. Sulfation reaction

ASP (500 mg) was suspended in anhydrous formamide (20 mL) at room temperature with stirring for 30 min, and the sulfating reagents were added dropwise. The mixture was stirred for 3 h at 60 °C. After the reaction, the mixture was cooled to room temperature and the pH value was adjusted to 7–8 with 2 mol/L NaOH solution. The mixtures were precipitated with EtOH (95%), washed, redissolved in water, and then dialyzed (molecular weight cutoff 8–12 kDa) against tap water for 48 h and distilled water for 24 h to remove pyridine, salt and potential degradation products. Five sulfated ASP (SASP-1 to SASP-5) with different DS were collected after lyophilizing and kept in dryness box.

The sulfur contents of SASP were determined by reported method (Wang, Li, et al., 2009). A calibration curve was constructed with sodium sulfate as standard. The degrees of substitution (DS) was calculated according to the equation:

$$DS = \frac{162 \times SO_4^{2-}\%}{100 - (96/98 \times SO_4^{2-}\%)} \quad (1)$$

### 2.3. Components analysis

The composition was analyzed according to the earlier report from our laboratory (Zhang et al., 2007). Briefly, 10 mL of sample was dissolved in 4 mL of 4 M trifluoroacetic acid acetate (TFA) in a test tube and then hydrolyzed at 120 °C for 10 h under air-tight condition. TFA was then evaporated through decompression and distillation. When the tube was dry, 10 mg of ammonium hydrochloride and 0.5 mg pyridine were added and allowed to react in a 90 °C water bath for 30 min. Then 0.5 mL cold (kept at 4 °C in a refrigerator) acetic anhydride was added to the test tube and the mixture was incubated in the 90 °C water bath for another 30 min to allow the acetylation reaction to occur. The end product was decompressed and distilled to dryness. The acetate derivatives were analyzed by GC–MS (Thermo Focus GC-Polaris Q MS, TR-5 ms SQC column, 30× 0.25 μm). The temperature program was set to increase from 120 to 250 °C at an increment of 5 °C/min and $N_2$ was the carrier gas. The standard monosaccharides were measured following the same procedure.

### 2.4. FT-IR analysis

FT-IR spectra were recorded with KBr pellets on Nicolet NEXUS 670 FT-IR. Sixteen scans at a resolution of 4 $cm^{-1}$ were averaged and referenced against air.

### 2.5. NMR spectroscopy

$^{13}C$ NMR experiments were recorded on a Bruker Avance DPX-400 spectrometer (operating frequency of 100.593 MHz). Samples were deuterium-exchanged several times by freeze drying from $D_2O$, and then examined in $D_2O$ at 25 °C. The chemical shifts were expressed in ppm relative to the resonance of the internal standard $Me_4Si$.

### 2.6. Molecular weight determination

HPSEC-LLS measurements were carried out on size-exclusion chromatograph combined with multi-angle laser photometer (MALLS, λ = 690 nm; DAWN EOS, Wyatt Technology Co., USA). Ultrahydrogel™ column (7.8× 300 mm, Waters, USA) was used as SEC instrument. An optilab refractometer (Dawn, Wyatt Technology Co., USA) was simultaneously connected. The polysaccharides

samples with desired concentrations were prepared and optical clarification of the samples was achieved by filtration into a scattering cell. The injection volume was 50 μL and the flow rate was 0.5 mL/min. The refractive index increment (d$n$/d$c$) value of the sample was determined by using an optilab refractometer at 690 nm (25 °C) to be 0.145 mL/g. The basic light scattering equation was as follows:

$$\frac{K_c}{R_\theta} = \frac{1}{M_w}\left(1 + \frac{16\pi^2\langle S^2\rangle_z}{3\lambda^2}\cdot\sin^2\left(\frac{\theta}{2}\right)\right) + 2A_2C \quad (2)$$

where $K$ was an optical constant equal to $[4\pi^2n^2(dn/dc)^2]/(\lambda^4N_A)$; $c$, the polysaccharide concentration in mg/mL; $R_\theta$, the Rayleigh ratio; $k$, the wavelength; $n$, the refractive index of the solvent; d$n$/d$c$, the refractive index increment; $N_A$, the Avogadro' number; $A_2$, the second virial coefficient. As the column separated the polymer according to molecular weight, each fraction was led to the light scattering detector for instantaneous measurement of the scattering intensities. The refractive index detector connected in series gave the polymer concentration. In chromatography mode, we had a single and sufficiently low concentration at a particular slice because of the further dilution by the SEC column of the already dilute injected sample.

### 2.7. Assay for antioxidant activities

#### 2.7.1. Superoxide radical scavenging assay

The superoxide radical scavenging assay was measured according to the method of Qi et al. (2005). Superoxide radicals were generated in a PMS/NADH system for being assayed in the reduction of NBT. Samples were dissolved in deionized water at the concentration of 0.01–2 mg/mL. The reaction mixture, containing varying concentrations of samples, Tris–HCl (16 mM, pH 8.0), NADH (338 μm), NBT (72 μm) and PMS (30 μm) was incubated at room temperature for 5 min and the absorbance was read at 560 nm against a blank. The capability to scavenge superoxide radical was calculated using the following equation:

$$\text{Scavenging effect}\,(\%) = \left[1 - \frac{A}{A_0}\right] \times 100\%$$

where $A_0$ was the absorbance of mixture solution without sample; $A$ was the absorbance of the test sample mixed with reaction solution.

#### 2.7.2. Hydroxyl radical scavenging assay

The hydroxyl radical assay was measured by the method of Ghiselli (Ghiselli, Nardini, Baldi, & Scaccini, 1998) with a minor modification. Samples were dissolved in deionized water at the concentration of 0.02–2.0 mg/mL. The sample solution (0.1 mL) was mixed with 0.6 mL of reaction buffer [20 mM phosphate buffer (pH 7.4), 2.67 mM deoxyribose, and 100 μm EDTA], 0.2 mL of 0.4 mM ferrous ammonium sulfate, 0.05 mL of 2.0 mM Vc, and 0.05 mL of 10 mM $H_2O_2$ was then added to the reaction solution. The reaction solution was incubated for 15 min at 37 °C and then 1 mL of 1% thiobarbituric acid (TBA) and 1 mL of 2% trichloroacetic acid (TCA) were added to terminate the reaction. The mixture was boiled for 15 min and cooled to room temperature. The absorbance of the mixture was measured at 532 nm against blank. The capability to scavenge hydroxyl radical was calculated using the following equation:

$$\text{Scavenging effect}\,(\%) = \left[1 - \frac{A}{A_0}\right] \times 100\%$$

where $A_0$ was the absorbance of mixture solution without sample; $A$ was the absorbance of the test sample mixed with reaction solution.

#### 2.7.3. Effect of scavenging 1,1-diphenyl-2-picryl-hydrazyl (DPPH) radicals

The free radical scavenging activity of the polysaccharides was measured by DPPH test according to the method of Shimada with some modifications (Shimada, Fujikawa, Yahara, & Nakamura, 1992). The 0.2 mmol/L solution of DPPH in methanol was prepared daily before UV measurements. One milliliter of the polysaccharides of different addition quantity (0.2–5 mg) in water was thoroughly mixed with 2 mL of freshly prepared DPPH and 2 mL of methanol. The mixture was shaken well, allowed to stand for 30 min in the dark, and the absorbance was then measured at 517 nm against a blank. Lower absorbance of the reaction mixture indicated higher free radical scavenging activity, which was analyzed from the graph plotted of inhibition percentage against compound concentration. BHA was used as positive controls. The experiment was carried out in triplicate and averaged. The capability to scavenge the DPPH radical was calculated using the following equation:

$$\text{Scavenging effect}\,(\%) = \left[\frac{A_0 - (A - A_b)}{A_0}\right] \times 100\%$$

where $A_0$ was the absorbance of DPPH solution without sample; $A$ was the absorbance of the test sample mixed with DPPH solution and $A_b$ was the absorbance of the sample without DPPH solution.

#### 2.7.4. Reducing power assay

The reducing power was determined according to the method of Qi et al. (2005). Different concentrations of samples (0.1–5 mg/mL, 2.5 mL) was mixed with 2.5 mL of 0.2 M sodium phosphate buffer (pH 6.6) and 2.5 mL of potassium ferricyanide (1%). The mixture was incubated at 50 °C for 20 min. The reaction was terminated by TCA solution (10%). Then, the solution was mixed with distilled water and ferric chloride (0.1%), the absorbance was measured at 700 nm against a blank. A higher absorbance indicated a higher reducing power. BHA was used for comparison.

#### 2.7.5. Metal chelating assay

The ferrous ion-chelating ability of polysaccharides extracted by MAE was investigated with slightly modified method of Li, Li and Zhou (2007). Samples in different concentrations (0.2–5 mg/mL) were mixed with $FeCl_2$ (0.1 mL, 2 mM) and ferrozine (0.4 mL, 5 mM), shook well, stayed still for 10 min at room temperature. Then the absorbance of the mixture was determined at 562 nm. In the control, sample was substituted with EDTA. The ferrous ion-chelating activity was given by the following equation:

$$\text{Chelating ability}\,(\%) = \left[\frac{(A_0 - A)}{A}\right] \times 100\%$$

where $A_0$ was the absorbance of mixture solution without sample; $A$ was the absorbance of the test sample mixed with reaction solution.

#### 2.7.6. Statistical analysis

All the data were shown in means ± S.D. within significance $p < 0.05$ after subjecting to an analysis of variance (ANOVA) and processed with SPSS 13.0.

## 3. Results and discussion

### 3.1. The sulfation of ASP

It was reported that controlling the reagent amount was better than controlling the reaction temperature to get sulfated polysaccharide derivatives with high DS (Liu et al., 2009). Five sulfated derivatives of ASP were obtained by varying the ratio of CSA to pyridine in the sulfating reagent (Table 1). The DS of the samples varied from 0.51 to 0.93. SASP-2 had the highest DS of 0.93 with the

**Table 1**
Sulfation of ASP with CSA/pyridine method.

| Samples | CSA:Pyr | Yield (mg) | Carbohydrate (%) | S (%) | DS |
|---|---|---|---|---|---|
| ASP | – | – | 90.30 | nd[a] | nd |
| SASP-1 | 4:1 | 0.421 | 28.17 | 8.80 | 0.62 |
| SASP-2 | 2:1 | 0.596 | 72.34 | 11.58 | 0.93 |
| SASP-3 | 1:1 | 0.579 | 66.48 | 10.68 | 0.82 |
| SASP-4 | 1:2 | 0.460 | 50.39 | 9.50 | 0.69 |
| SASP-5 | 1:4 | 0.488 | 42.51 | 7.62 | 0.51 |

[a] Not detected.

**Table 2**
Monosaccharide composition (molar ratio) of ASP and its sulfated derivative by GC–MS.

| Samples | Ara | Xyl | Lyx | Man | Glu | Gal |
|---|---|---|---|---|---|---|
| ASP | 1 | 1.6 | 1.74 | 27.6 | 3.9 | 14.4 |
| SASP-1 | 1 | 6.64 | –[a] | 12.7 | 23.3 | 5.8 |
| SASP-2 | 1 | 6.66 | – | 12.8 | 19.6 | 7.19 |
| SASP-3 | 1 | 8.24 | – | 12.5 | 22.4 | 9.01 |
| SASP-4 | 1 | 11.57 | – | 20.18 | 24.5 | 6.5 |
| SASP-5 | 1 | 9.4 | – | 22.8 | 29.3 | 9.9 |

[a] Not detected.

yield of 596 mg. With the decrease of the ratio of CSA to pyridine from 4:1 to 1:4, the DS of the corresponding derivates decreased. A moderate DS and lowest yield of SASP-1 indicated that too much of CSA had negative effect on sulfated derivative. This was in accordance with the previous studies that it could cause hardening and uneven reaction in practical manipulation if the proportion of CSA was over high (Wang, Li, et al., 2009).

It was reported that the antiviral activity of sulfated polysaccharides was direct correlation with carbohydrate content (Lu et al., 2008). The result was in accordance with the previous studies that the carbohydrate content was decrease in all sulfated samples (Sun et al., 2009). The carbohydrate content was varying from 18.34% to 78.56% and 21.07% to 62.47% in sulfated rice bran and epimedium polysaccharides, respectively (Lu et al., 2008; Wang, Li, et al., 2009; Wang, Zhang, Zhang, Zhang, Li, 2009).

Analysis of monosaccharide composition of SASP showed that these polysaccharides consisted of arabinose, xylose, mannose, glucose, galactose, which accounted for the majority of monosaccharides present (Table 2). In all sulfated samples, glucose was the dominant monosaccharide and lyxose was not detected. The monosaccharide composition of SASP-2 with the highest DS was showed in Fig. 2. In contrast, ASP had much higher mannose content, making it the predominant monosaccharide. The difference between ASP and its sulfated derivative might be due to the degradation of backbone and side chain in the sulfation process.

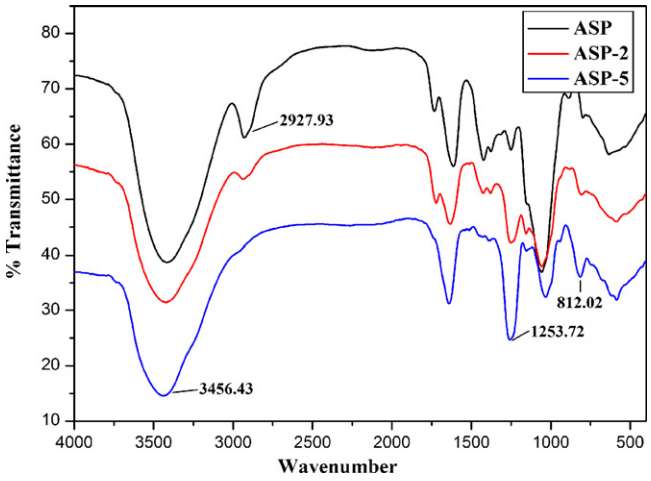


**Fig. 1.** FT-IR spectra of ASP and its sulfated derivatives with different DS.

### 3.2. FT-IR analysis

Fig. 1 showed the FT-IR spectra of ASP, SASP-2 (DS = 0.93) and SASP-5 (DS = 0.51). The bands around 3450 and 2927 $cm^{-1}$ were due to the hydroxyl stretching vibration and C–H stretching vibration, respectively. With the increasing of DS, the intensity of 2927 $cm^{-1}$ was decreased. New bands at 1253.72 and 1251.79 $cm^{-1}$

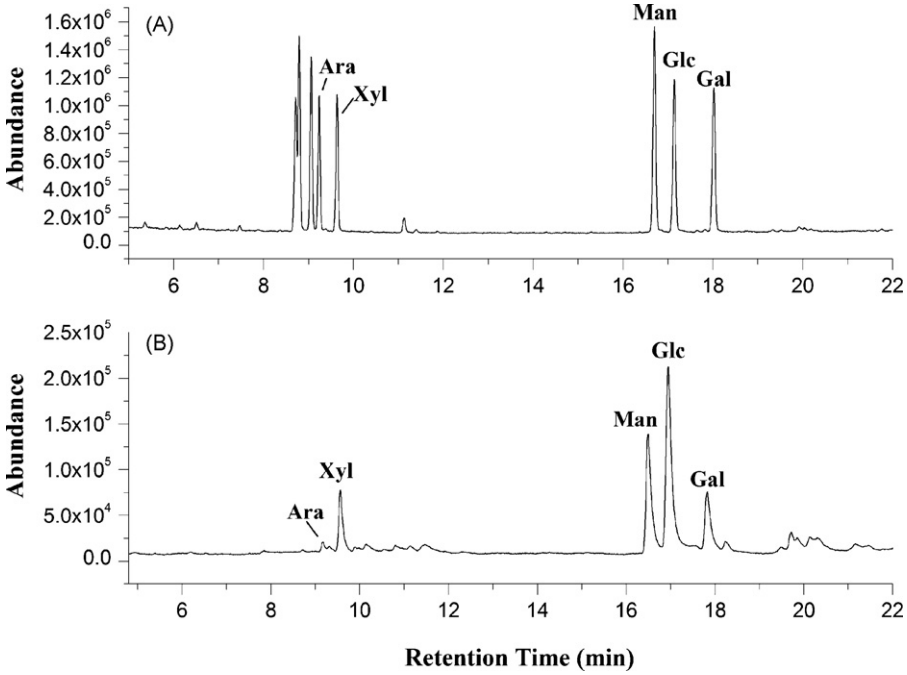


**Fig. 2.** GC–MS of standard monosaccharides (A) and SASP-2 (B).

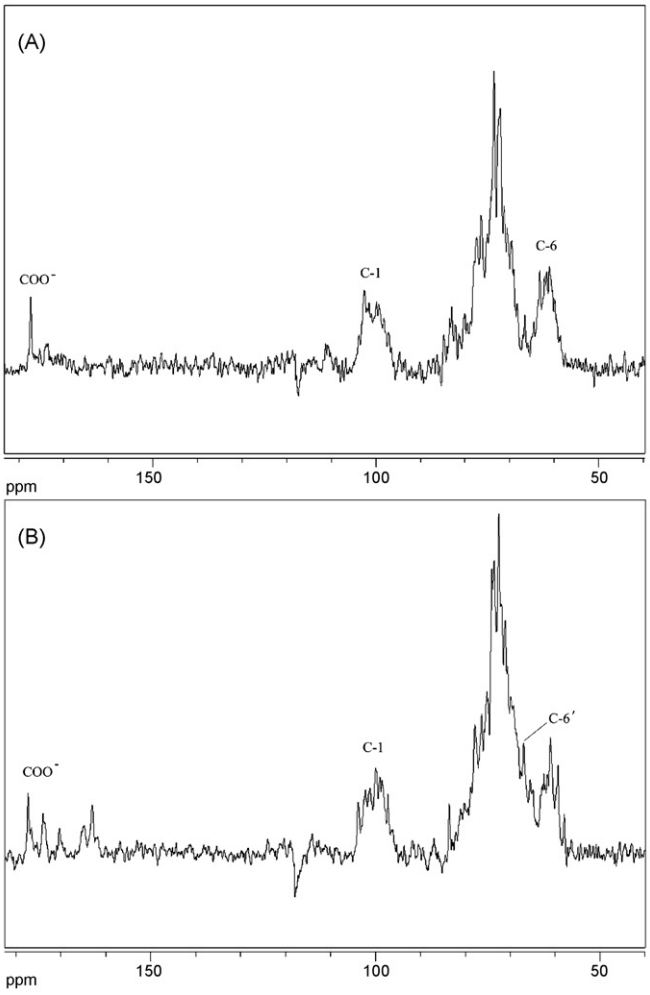


**Fig. 3.** $^{13}$C NMR spectra of ASP (A) and its sulfated derivatives, SASP-2 (B).

describing an asymmetrical S=O stretching vibration in SASP samples. The region around 800–850 cm$^{-1}$ was used to infer the position of the sulfate group in sulfated polysaccharides. The bands at 845, 830 and 820 cm$^{-1}$ were assigned to the 3-sulfate, 2-sulfate and 6-sulfate, respectively (Jeanny et al., 2008). SASP-2 and SASP-5 showed a moderate intensity bands at 812.02 and 815.88 cm$^{-1}$ indicating a symmetrical C–O–S vibration which was attributed to the sulfate substitution at the C-6 of galactose. It was obvious that reactivity of ASP hydroxyl group was C-6. The low reactivity of position C-2 and C-3 could be attributed to the steric hindrance (Yang, Du, et al., 2005; Yang, Gao, et al., 2005).

### 3.3. $^{13}$C NMR

The $^{13}$C NMR spectra of SASP-2 were presented in Fig. 3B, where the signal positions were compared to that of ASP (Fig. 3A). As shown in Fig. 3A, the signals at 103.7, 102.5 and 99.7 ppm were assigned to the anomeric carbons (C-1) of L-rhamnose, β-D-galactose linked to α-L-galactose (Jeanny et al., 2008; Yang, Du, Huang, Wan, & Li, 2002; Zou et al., 2008) and β-D-mannopyranosyl (Marco, Miguel, Mutue, Rosiane, & Maria, 2007), respectively. Signals at 82.9 and 77.3 ppm were assigned to C-3 and C-4 of β-D-mannopyranosyl (Nina et al., 2004). The signal at 76.4 ppm was assigned to β-D-xylose linked to C-6 of the β-D-galactose units (Maria & Maria, 2003). The signals of unlinked C-6 were at 63.2, 61.7 and 61.0 ppm, the signals of linked C-6 were at 69.6 and 71.2 ppm. The integration of the latter was much less than that of the former. These indicated that the polysaccharides had a structure of branches (Yang et al., 2002). In addition, the signal at 177.4 ppm was assigned to D-glucuronic acid (Lu & Yoshida, 2003; Miguel et al., 2008; Yang, Du, et al., 2005; Yang, Gao, et al., 2005; Yang et al., 2002).

It was found that the $^{13}$C NMR spectra became more complicated after sulfation because the carbon directly attached to an electron-withdrawing sulfate group would shift to a lower field position, while the carbon indirectly attached to sulfate group would shift to higher field position (Liu et al., 2009; Yang, Du, et al., 2005; Yang, Gao, et al., 2005). The new peak at 67.0 ppm of SASP was assigned to the O-6 substituted carbons, suggesting sulfation of O-6 (Liu et al., 2009; Marco et al., 2007; Melo, Feitosa, Feitosa, Freitas, & Paula, 2002). The signals of the anomeric carbon was split into two peaks at 69.9 and 69.2 ppm were assigned to the O-6 substituted carbons, which also a result of sulfation. C-6 peaks attributed to β-D-galactose linked to α-L-galactose still remained at 62.5, 61.6 and 61.0 ppm for SASP, suggesting that the primary OH groups on the internal side of the helix were not sulfated (Liu et al., 2009). It was known that the signal of C-1 splits if OH group on C-2 was functionalized and this splitting of the C-1 signal correlates well with the extent of substitution at the C-2 atom (Liu et al., 2009; Yang, Du, et al., 2005; Yang, Gao, et al., 2005). SASP showed a split of the signals at 99–103 ppm for C-1. Obviously, all can be assigned for C-1 either without a sulfate group on C-2 (102.2 ppm) or for C-1 with a sulfate group on C-2 at 99.9, 99.7 (β-D-galactose O-6 substitution) and 98.5 ppm. In addition, the position at 98.5 ppm was assigned to the signal of C-1 with sulfate substitution at C-6 and C-2 in the glucose residue (Han, Yao, Yang, Liu, & Gao, 2005).

From the results of $^{13}$C NMR, the non-selective sulfation of ASP occurred, and the intensity of the signals of the O-substitution carbons denoted that C-6 substitution was predominant in SASP compared with other positions at C-2, probably owing to steric hindrance as reported elsewhere (Yang, Du, et al., 2005; Yang, Gao, et al., 2005).

**Table 3**
Molecular characterization of ASP and its sulfated derivative by HPSEC-LLS.

| Samples | $M_w \times 10^4$ | $M_n \times 10^4$ | $M_z \times 10^5$ | $M_w/M_n$ | $\langle S^2\rangle_z^{1/2}$ (nm) |
|---|---|---|---|---|---|
| ASP | 7.348 | 2.308 | 71.41 | 3.184 | 29.9 |
| SASP-1 | 0.441 | 0.321 | 0.908 | 1.372 | 30.7 |
| SASP-2 | 3.203 | 1.581 | 9.980 | 2.025 | 35.8 |
| SASP-3 | 2.110 | 0.6172 | 4.083 | 3.719 | 36.9 |
| SASP-4 | 1.353 | 0.3789 | 6.951 | 3.571 | 31.9 |
| SASP-5 | 0.645 | 0.419 | 2.317 | 1.539 | 34.1 |

### 3.4. Analysis of molecular properties

The characterization of natural polysaccharides and its derivatives having various chemical components, molar mass and chain conformation was important because of their critical effect on end-use structure–property relations (Cui et al., 2008). The molecular weight and chain conformation of ASP and its sulfated derivates were determined with SEC-LLS. The weight average molar mass ($M_w$), polydispersity (PD, $M_w/M_n$) and $z$-average radius of gyration ($\langle S^2\rangle_z^{1/2}$) were shown in Table 3 for all the samples. The angular dependences of $\left(K_C/R_\theta\right)^{1/2}$ for ASP and its sulfated derivates were shown in Fig. 4A. The radius of gyration, $M_w$, and PD for ASP were measured to be 29.9 nm, 7.348 × 10$^4$, and 3.184, respectively.

Our results showed that five sulfated derivates with different DS and molecular weight was obtained by varying the ratio of CSA to pyridine. The $M_w$ values and DS of sulfated samples decreased with the increasing of pyridine contents. However, too much of CSA (ratio of 4:1) resulted in the lowest $M_w$ value in SASP-1. Compared to the native one, all the samples showed a sharp decrease in weight

**Table 4**
Molecular properties of ASP and its sulfated derivates.

| Samples | Relation equation | Exponents | $d_f$ | Conformation |
|---|---|---|---|---|
| ASP | $\langle S^2\rangle_z^{1/2} = 0.203M_w^{0.35\pm0.027}$ | 0.35 | 2.86 | Between hard sphere and random coil (fully swollen) |
| SASP-1 | $\langle S^2\rangle_z^{1/2} = 0.307M_w^{0.45\pm0.061}$ | 0.45 | 2.22 | Between hard sphere and random coil (not swollen) |
| SASP-2 | $\langle S^2\rangle_z^{1/2} = 0.141M_w^{0.42\pm0.044}$ | 0.42 | 2.38 | Between hard sphere and random coil (not swollen) |
| SASP-3 | $\langle S^2\rangle_z^{1/2} = 0.286M_w^{0.48\pm0.049}$ | 0.48 | 2.08 | Between hard sphere and random coil (not swollen) |
| SASP-4 | $\langle S^2\rangle_z^{1/2} = 0.261M_w^{0.51\pm0.033}$ | 0.51 | 1.96 | Random coil |
| SASP-5 | $\langle S^2\rangle_z^{1/2} = 0.309M_w^{0.36\pm0.034}$ | 0.36 | 2.77 | Between hard sphere and random coil (fully swollen) |

average molecular weight and more broad molar mass distribution due to the extensive degradation of the polysaccharides during the sulfation. This might be due to the hydrolysis of polysaccharide in acid environment.

Relevant structural information and further insight into the nature of the polysaccharide can be obtained by investigating the fractal dimension ($d_f$) (Tao, Zhang, & Peter, 2006). The $d_f$ value could be determined from the $M_w$ dependence of $\langle S^2\rangle_z^{1/2}$, and was defined as the inverse of the exponent $\nu$:

$$\langle S^2\rangle_z^{1/2} = fM^{\nu} \quad (3)$$

$$d_f = \frac{1}{\nu} \quad (4)$$

On the basis of the theory of polymer solutions, the value of $d_f$ was 1 for a rigid rod, and linear polymers with Gaussian coil nature had $d_f$ value ranging from 5/3 to 2. A three-dimensional object with a homogeneous density had a mass fractal dimension of 3. The $d_f$ value of monodisperse polymers could be extracted directly from the angular dependence of the scattered light or neutron intensity. This approach had been successfully applied to highly branched polysaccharides with high molecular size such as amylopectin, and other synthetic branched polymers (Tao & Xu, 2008).

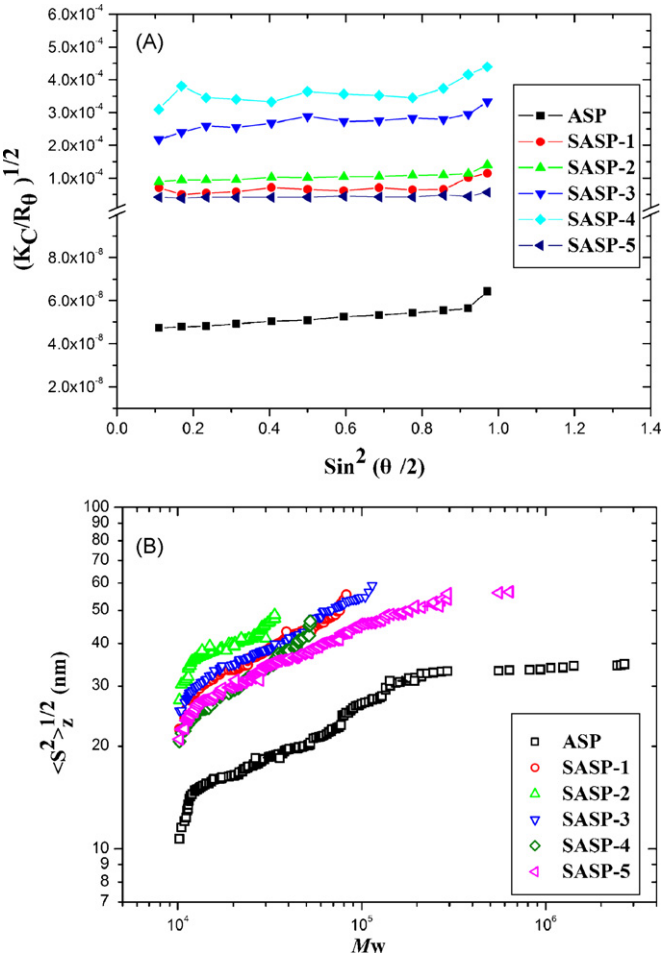


**Fig. 4.** Plot of the angular dependences of $(K_C/R_\theta)^{1/2}$ (A) and log $\langle S^2\rangle_z^{1/2}$ versus log $M_w$ (B) for ASP and its sulfated derivates at 25 °C.

Fig. 4B showed the plot of $M_w$ versus $\langle S^2\rangle_z^{1/2}$ of ASP and its sulfated derivates. The straight line fitting of the experimental points was shown in Table 4. The $\nu$ value of 0.35 suggested that ASP molecules in aqueous solution were in the state between hard sphere and random coil. Furthermore, the value of $d_f$ was calculated to be 2.86 according to Eq. (4). The $d_f$ value of 2.86 was characteristic of a particle having an internal structure between the hard sphere ($d_f$ = 3.0) and the fully swollen branched macromolecule in a thermodynamically good solvent ($d_f$ = 2.0) (Tao et al., 2006). The result confirmed that ASP existed as a sphere conformation of branched clusters in aqueous solution. In sulfated samples, a decrease in $d_f$ values was observed. The $d_f$ values for SASP-1, SASP-2 and SASP-3 were 2.22, 2.38 and 2.08, respectively. A $d_f$ value of 2.08–2.38 was characteristic of a particle having an internal structure between hard sphere and the branched macromolecules which were not swollen, either for thermodynamic reasons or because of steric hindrances (Bauer & Burchard, 1993). However, the $\nu$ value of 0.51 for SASP-4 was larger than all of the samples. The $d_f$ value of 1.96 indicated that SASP-4 exhibited as a random coil conformation in aqueous solution.

Our results indicated that sulfated polysaccharides showed less flexibility compared with native one. However, no obvious relationship between DS and chain conformation parameters was observed. It was confirmed by the results of Tao et al. Similar $d_f$ value (1.79) in sulfated polysaccharide showed that the $-SO_3H$ groups in the derivatives enhance the steric hindrance between the polymer chains, leading to the relatively expanded conformation of the sulfated derivatives (Tao et al., 2006).

The molecular property of sulfate polysaccharide was an important parameter influencing bioactivity. Based on the results, it could be concluded that sulfation and degradation occur simultaneously in the sulfation process (Han et al., 2005; Parvathy et al., 2005; Yang, Du, et al., 2005; Yang, Gao, et al., 2005). The mechanism of degradation was regarded as hydrolysis, so the removal of residual water became an essential step in the sulfation of ASP (Zou et al., 2008). In addition, conformation transition was observed in the sulfation process.

### 3.5. Antioxidant activity analysis

#### 3.5.1. Scavenging activity of superoxide radical

The scavenging ability of superoxide anion radicals is extremely important to anti-oxidation work. It is known to indirectly initiate lipid peroxidation as a result of $H_2O_2$ formation, creating precursors of hydroxyl radicals. In the PMS/NADH-NBT system, superoxide anion derived from dissolved oxygen by the PMS/NADH coupling reaction reduces NBT (Xu et al., 2009).

Fig. 5A showed that the inhibitory effect of all different sulfate content samples on superoxide radical was significant at all

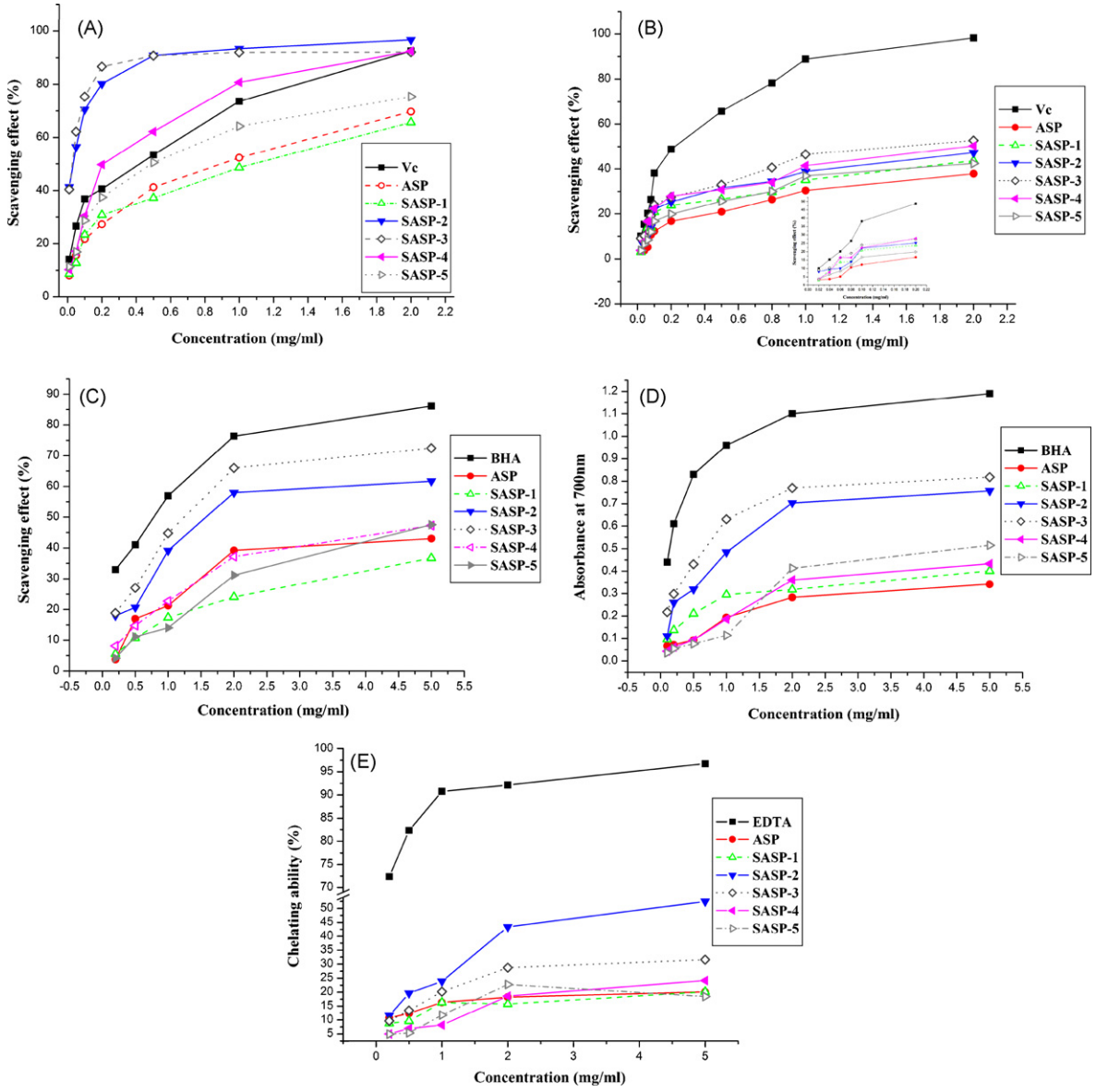


**Fig. 5.** Antioxidant effect of ASP and its sulfated derivatives: (A) scavenging activity of superoxide radicals; (B) scavenging activity of hydroxyl radicals; (C) scavenging activity of DPPH radicals; (D) reducing power; (E) chelating effect on ferrous ions; data are presented as mean values ($n = 3$).

tested concentrations and in a concentration dependent manner. The $EC_{50}$ values of ASP, SASP-1, SASP-2, SASP-3, SASP-4 and SASP-5 were 1.12, 1.22, 0.069, 0.076, 0.57 and 0.89 mg/mL, respectively. Similar results from Zhang et al. reported that the $EC_{50}$ value of three sulfated *Porphyra haitanensis* polysaccharides was between 0.06 and 1.6 mg/mL (Zhang et al., 2003). In addition, sulfated polysaccharides from *Laminaria japonica* and *Ulva pertusa* showed the best $EC_{50}$ values of 1.7 and 5.8 μg/mL, which was higher than $EC_{50}$ value in the present study due to the higher sulfate content (Qi et al., 2005; Wang, Zhang, Zhang, & Li, 2008). At a concentration of 0.5 mg/mL, the scavenging effect was 90.83% and 90.7% for SASP-2 and SASP-3, respectively. At the same concentration, the scavenging effect of Vc was only 53.4%. Compared to the results, all sulfated samples showed stronger scavenging effect than ASP except for SASP-1 while SASP-2, SASP-3 and SASP-4 had stronger scavenging effect than Vc. This demonstrated that DS affected the antioxidant activity, which was in accordance with Wang et al. that higher sulfate content showed greater scavenging effect of superoxide radical (Wang et al., 2008). Our data on the activity of scavenging superoxide radicals suggested that it was likely to contribute towards the observed antioxidant effect.

### 3.5.2. Scavenging activity of hydroxyl radical

Hydroxyl radicals, generated by reaction of iron-EDTA complex with $H_2O_2$ in the presence of Vc, attack deoxyribose to form products that, upon heating with 2-thiobarbituric acid under acid conditions, yield a pink tint. Added hydroxyl radical scavengers compete with deoxyribose for the resulted hydroxyl radicals and diminish tint formation (Qi et al., 2005). As shown in Fig. 5B, all the sulfated samples were found to have the ability to scavenge hydroxyl radicals in a concentration-dependent fashion. SASP-3 showed the best scavenging ability, while the original ASP showed a very low scavenging effect. The $EC_{50}$ values were 2.1, 1.82, 1.52, 1.69 and 2.05 mg/ml for SASP-1 to SASP-5, respectively. The result indicated that the sulfate group played an important role in the scavenging of hydroxyl radicals. This was in accordance with the result of Qi et al. that in the molecule of high DS, part of –OH groups were substituted by $–OSO_3H$ groups, so the scavenging effect enhanced (Qi et al., 2005). Although SASP-2 had higher DS

than SASP-3, the scavenging ability of SASP-2 was weaker than SASP-3. This could be due to the molecule weight of sulfated samples. Zou et al. confirmed that moderate $M_w$ ($1.27 \times 10^4$) and DS (0.34) showed the best antioxidant capacities (Zou et al., 2008).

It was reported that the scavenging activity of hydroxyl radical was due to the inhibition of hydroxyl radical generation by chelating ions such as $Fe^{2+}$ and $Cu^{2+}$ (Zou et al., 2008). Hydroxyl radicals can be generated by the reaction of $Fe^{2+}$ and $H_2O_2$, and since the sulfate group of sulfated ASP had chelating ability for $Fe^{2+}$, this sulfated polysaccharide could reduce the generation of hydroxyl radicals by chelating the $Fe^{2+}$. In another assay system in this study, it was demonstrated that the iron chelating ability and the trend of the chelating ability was nearly the same to the order of the scavenging ability to hydroxyl radical. The antioxidant activities of SASP were not a function of a single factor but a combination of several factors (Wang et al., 2008).

*3.5.3. Scavenging activity of DPPH radicals*

DPPH is a stable free radical that shows maximum absorption at 517 nm in methanol. The model of scavenging the stable DPPH radical is a widely used method to evaluate the free radical scavenging ability of natural compounds (Chen, Zhang, et al., 2008; Chen, Xie, et al., 2008). When DPPH encounters a proton-donating substance, for example, an antioxidant, the radical would be scavenged and the absorbance at 517 nm is reduced. Based on this principle, the antioxidant activity of a substance can be expressed as its ability in scavenging the DPPH free radical. In the DPPH test, the antioxidants were able to reduce the stable DPPH radical to the yellow-coloured diphenylpricrylhydrazine. The scavenging ability of the samples on DPPH radical was shown in Fig. 4C and compared with BHA.

Both SASP-2 and SASP-3 had strong antioxidant activity, the scavenging effects were 61.71% and 72.45% at a dose of 5 mg/mL, higher than ASP but lower than BHA. The scavenging effects were 36.72%, 47.24% and 47.52% for SASP-1 SASP-4 and SASP-5 at the concentration of 5 mg/mL, which were weaker than native ASP. This could be due to the over degradation of polysaccharides molecule during sulfation. It was reported that the effect of antioxidant was due to their hydrogen donating ability (Wang, Zhang, et al., 2009). The presence of $-OSO_3H$ groups in the SASP molecule could activate the hydrogen atom of the anomeric carbon. The higher activated capacity of the group, the stronger hydrogen atom-donating capacity. It was concluded that SASP-3 with high DS and moderate $M_w$ showed the best scavenging activity of DPPH radicals.

*3.5.4. Reducing power*

The presence of a reluctant such as antioxidant substances in the antioxidant samples causes the reduction of the $Fe^{3+}$/ferricyanide complex to the ferrous form. Therefore, $Fe^{2+}$ can be monitored by measuring the formation of Prussian blue at 700 nm (Qi et al., 2006). Fig. 5D depicted the reducing power of the samples and BHA. The reducing power values of SASP-1 to SASP-5 were 0.295, 0.484, 0.63, 0.187 and 0.114 at 1 mg/mL, which were weaker than BHA. Moreover, the reducing power of SASP was relating more pronounced than that of native one and SASP-3 was the most pronounced. Wang et al. reported that the reducing power of sulfated polysaccharides from *Laminaria japonica* was between 0.079 and 0.106 at the concentration of 1 mg/mL (Wang et al., 2008). The reducing power of sulfated lacquer polysaccharides (LPS5) was 1.2 at 1 mg/mL (Zou et al., 2008). Compared to the results, sulfated ASP had strong reducing capacity and the relation of reducing power to DS was significant.

The reducing properties were generally associated with the presence of reductones, which had been shown to exert antioxidant action by breaking the free radical chain by donating a hydrogen atom (Qi et al., 2005). Reductones were also reported to react with certain precursors of peroxide, thus preventing peroxide formation. Our data on the reducing power of sulfated ASP suggested that it was likely to contribute towards the observed antioxidant effect.

*3.5.5. Chelating effect on ferrous ions*

Ferrum is known as the most important lipid oxidation prooxidant due to its high reactivity. The ferrous state of ferrum accelerates lipid oxidation by breaking down hydrogen and lipid peroxidase to reactive free radicals. $Fe^{3+}$ also produces radicals from peroxides, although the rate is tenfold less than that of $Fe^{2+}$. Ferrozine can quantitatively form complexes with $Fe^{2+}$. In the presence of other chelating agents, the complex formation is disrupted with the result that the red color of the complexes decreases. Measurement of color reduction therefore allows estimating the metal chelating activity (Qi et al., 2006).

The ferrous ion chelating effects of SASP-2 and SASP-3 were concentration related and those of SASP-1, SASP-4 and SASP-5 were not concentration dependent as shown in Fig. 5E. Relationship between chelating effect and DS was obvious. At the concentration of 5 mg/mL, SASP-2 and SASP-3 showed the highest chelating abilities of 52.49% and 31.59%, respectively. At low concentration, the chelating ability of ASP was stronger than that of SASP-1, SASP-4 and SASP-5, but it was not significant. At the concentration above 2 mg/mL, the effects of sulfated derivates were more pronounced. It was reported that the chelating effect of sulfated *Laminaria japonica* polysaccharides was 18.97–29.58% at the concentration of 0.76–1.18 mg/mL. Compared with the results, sulfated ASP showed moderate chelating ability on ferrous ions. The present study was in accordance with previous results that the substitution of –OH with $-OSO_3H$ groups would enhance the ferrous chelating ability (Yang, Du, et al., 2005; Yang, Gao, et al., 2005).

The antioxidant mechanism may be due to the supply of hydrogen by SASP, which combines with radicals and forms a stable radical to terminate the radical chain reaction. The other possibility is that SASP can combine with the radical ions which are necessary for radical chain reaction and then the reaction is terminated. However, the exact explanation of mechanism underlying the free radical scavenging activity exerted by polysaccharides is still not fully understood (Chen, Zhang, et al., 2008; Chen, Xie, et al., 2008).

## 4. Conclusion

In the present study, five sulfated derivatives of ASP with different DS were synthesized. The structure and corresponding antioxidant activities were evaluated in vitro. Two characteristic absorption bands appearance in FT-IR spectra near 1250 and 810 $cm^{-1}$ indicating the sulfation reaction had occurred. From the results of $^{13}C$ NMR, the non-selective sulfation of ASP occurred, and the intensity of the signals of the O-substitution carbons denoted that C-6 substitution was predominant in SASP compared with other positions at C-2.

Compared with ASP, a sharp decrease in $M_w$ was observed in SASP. The changes in $d_f$ values showed a conformation transition in sulfation reaction. The $d_f$ values from 1.96 to 2.77 indicated that the $-SO_3H$ groups in the derivatives enhance the steric hindrance between the polymer chains, leading to the relatively expanded conformation of the sulfated derivatives.

Sulfated derivatives of ASP showed greater antioxidant activities compared to native one. It was obvious that DS had significant effect on the antioxidant activity. It was concluded that high DS and moderate $M_w$ could promote the antioxidant activities. The antioxidant mechanisms of SASP might be attributed to strong hydrogen donating ability, a metal chelating ability, and their effectiveness as scavengers of superoxide and free radicals. Overall, the present experiments on bioactivity of SASP showed that it was useful as functional food as well as potential therapeutic agent.

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