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Structural characterization, sulfation and antitumor activity of a polysaccharide fraction from *Cyclina sinensis*

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ABSTRACT

In the present study, we investigated the preliminary structure, sulfation and antitumor activity of a polysaccharide fraction from *Cyclina sinensis* (CSPS-1). Results of structural characterization showed that the backbone chain of CSPS-1 was composed of glucose linked by α-(1 → 4) glycosidic bond, and the branch chain was attached to backbone chain by (1 → 6) glycosidic bond. CSPS-1 was sulfated by chlorosulfonic acid-pyridine method under different modification conditions according to the orthogonal test $L_9(3^4)$, affording nine sulfated polysaccharides (CSPS-1-S). The optimal sulfation conditions for CSPS-1 were reaction temperature of 65 °C, reaction time of 2 h and chlorosulfonic acid-pyridine ratio of 1:4. Structural analysis revealed that sulfation had occurred at position of C-6 in CSPS-1. In addition, CSPS-1-S exhibited significantly higher inhibitory activity *in vitro* against human gastric cancer BGC-823 cells.

1. Introduction

In the last decades, much attention has focused on the biological properties of polysaccharides and their chemical derivatives, such as sulfated, phosphorylated and acylated derivatives (Shi, Nie, Chen, Liu, & Tao, 2007; Xing et al., 2005). Sulfation of polysaccharides can enhance water solubility, resulting in alteration of their biological activities (Wang et al., 2010). It has been reported that sulfated polysaccharides exhibit potent biological properties in comparison with non-sulfated polysaccharides, such as anti-viral, anti-oxidant and anti-tumor activities (Chen et al., 2011; Karmakar, Pujol, Damonte, Ghosh, & Ray, 2010; Liu, Wan, Shi, & Lu, 2011; Zou, Du, Li, Yang, & Zhang, 2010). Therefore, sulfation can be considered as an effective way to enhance the biological activities of polysaccharides.

Cyclina sinensis, a bivalve mollusk in the family of Veneridae, has been used as a traditional Chinese medicine for several centuries (Liu, Zhang, Dou, Lu, & Guo, 1997). It has been demonstrated that it is rich in protein, lipid and polysaccharides that may contribute to its biological functions, such as anti-tumor, anti-inflammation and immune-regulation (Abdulkadir & Tsuchiya, 2008; Gu, Yu, & Cai, 2006; Jiang, Wang, Liu, Gan, & Zeng, 2011; Jiang et al., 2013; Liu et al., 1997). Recently, we reported the purification and antitumor activity *in vitro* of polysaccharides from *C. sinensis* (CSPS) (Jiang et al., 2011). We found that the purified fractions of CSPS had potential antitumor activity. Among those purified fractions, CSPS-1, a neutral polysaccharide with a low content of sulfuric radical (Jiang et al., 2011), comprised the largest part of CSPS, but its antitumor activity was lower. This might limit its application. Therefore, modification of CSPS-1 by sulfation may be an effective way to enhance the antiproliferative activity of CSPS. It has been reported that many factors such as molecular weight, chain conformation, charge characteristics and position of substitution of polysaccharide may influence its antitumor activity (Ma et al., 2013). However, little attention has been shown to the chemical structure of CSPS. In addition, the relationship between structure and activity of CSPS remains unclear due to lack of structural data. Therefore, we report here the structural characterization, sulfation and antitumor activities *in vitro* of sulfated derivatives of CSPS-1. First, the structure of CSPS-1 was characterized by using nuclear magnetic resonance (NMR), Fourier-transform infrared (FT-IR) spectroscopy, periodic acid oxidation, Smith degradation and methylation combined with gas chromatography-mass spectrometry (GC–MS). CSPS-1 was then sulfated by chlorosulfonic acid-pyridine (CSA-Pyr) method, resulting in nine sulfated derivatives (CSPS-1-S). Finally, their antitumor activities *in vitro* on human gastric cancer BGC-823 cells were evaluated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT)-based colorimetric method.

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2. Materials and methods

2.1. Materials and reagents

A purified fraction of CSPS (CSPS-1) was prepared from *C. sinensis* according to our reported method (Jiang et al., 2011). Human gastric cancer BGC-823 cells were obtained from the Cell Bank of Shanghai Institute of Cell Biology (Shanghai, China). MTT, monosaccharide references (arabinose, rhamnose, fucose, xylose, galactose, glucose and mannose) and glucan reference were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Fetal bovine serum and RPMI-1640 media were purchased from Gibco/Invitrogen (Grand Island, NY, USA). All other reagents were analytical grade.

2.2. Structural characterization of CSPS-1

2.2.1. FT-IR spectrometric analysis

FT-IR spectrum of polysaccharide samples was recorded with a Nicolet 6700 FT-IR spectrometer (Thermo Fisher Scientific Inc., MA, USA) using potassium bromide (KBr) disks method (Wang et al., 2004). Briefly, samples were dried at 35–44 °C in vacuum over P_2O_5 for 48 h, ground with KBr powder and then pressed into pellets for FT-IR spectral measurement in the frequency range of 4000–400 cm^{-1}.

2.2.2. Periodic acid oxidation, Smith degradation and gas chromatographic analysis

Periodic acid oxidation, Smith degradation and gas chromatography (GC) analysis of CSPS-1 were performed according to reported methods with some modifications (Ghosh et al., 2008; Li et al., 2008; Rout, Mondal, Chakraborty, & Islam, 2008). Briefly, sodium metaperiodate solution (0.015 M) and sodium iodate solution (0.015 M) were prepared, respectively and mixed at ratios of 5:0, 4:1, 3:2, 2:3, 1:4 and 0:5. The mixed solution (0.1 ml) was diluted to 25 ml and the absorbance (Abs) was measured at 223 nm. A standard curve of $NaIO_4$ was plotted by using concentration of $NaIO_4$ as *X*-axis and Abs at 223 nm as *Y*-axis.

For determination of the amount of $NaIO_4$ consumed during the periodic acid oxidation, 20 mg polysaccharide was added to the solution of 40 ml 0.015 M $NaIO_4$. The reaction mixture was gently stirred, then kept in the dark at 4 °C for 2 h. Then, 0.1 ml of reaction mixture was diluted to 25 ml and the Abs of diluted solution was determined at 223 nm every 24 h until a constant optical density was observed. Ethylene glycol (1.0 ml) was added to the reaction system with stirring for 30 min to decompose the excess $NaIO_4$. The consumed amount of $NaIO_4$ was calculated according to the $NaIO_4$ standard curve.

Production of formic acid was determined by titration. One milliliter of the reaction product was mixed with 50 μl of phenolphthalein solution (0.5%), and 0.5 mM NaOH was then added drop by drop until the color of the reaction system changed from colorless to purple red. Amount of formic acid was calculated according to the amount of NaOH.

Finally, the rest of the reaction product was reduced by adding 50 mg of sodium borohydride ($NaBH_4$) for about 20 h and was then adjusted to pH 5.5–7.0 by using 0.1 M acetic acid. The reaction solution was dialyzed against running water and distilled water each for 24 h. The dialyzed product was lyophilized and 5 mg of the dried powder was hydrolyzed with 2 ml of 2 M trifluoroacetic acid at 120 °C for 2 h. The hydrolyzate was repeatedly co-distilled with methanol to dryness and conventionally converted into alditol acetates. The alditol acetate derivatives of standard monosaccharides, glycerol and erythritol were prepared in the same way. Then, all the derivatives were analyzed by a 6890N GC (Agilent Technologies, Inc., Santa Clara, CA, USA) equipped with flame ionization detector and an HP-5 fused silica capillary column (30 m × 0.32 mm × 0.25 μm). The operation conditions of GC were as follows: flow rates of N_2, H_2 and air were 25, 30 and 400 ml/min, respectively; the temperatures of detector and inlet were set at 280 and 250 °C, respectively; initial column temperature was held at 120 °C for 3 min, then programmed to 210 °C at a rate of 3 °C/min and held at 210 °C for 4 min.

2.2.3. Methylation and GC–MS assay

Methylation and GC–MS assay were performed by using the reported procedure (Ghosh et al., 2008; Mondal et al., 2008) with slight modifications. Ten milligram of polysaccharides sample was dissolved in 2 ml dimethyl sulfoxide (DMSO) in a 15 ml three-necked flask. After 50 mg of dried NaOH power was added, the mixture was agitated by a magnetic stirrer under the protection of N_2. Half an hour later, 1.0 ml of methyl iodide was added to the reaction system under the same condition. Half an hour later again, the reaction was stopped by adding 0.5 ml of distilled water, and the reaction solution was dialyzed against running water for 48 h and distilled water for 24 h. The dialyzed product was freeze-dried to obtain partially methylated polysaccharides. Completely methylated polysaccharide, confirmed by the disappearance of O—H absorption band (3700–3100 cm^{-1}) in FT-IR spectrum, was gained by repeating the methylation procedures mentioned above three times. The completely methylated polysaccharide (5 mg) was hydrolyzed with 2 ml of 2 M trifluoroacetic acid and acetylated as described above. The acetylated derivative was filtered through 0.22 μm of nylon membrane and analyzed by GC–MS (Satum 2200 GC–MS, Varian) with quartz capillary column (DB-5MS, 30 m × 0.25 mm × 0.25 μm). The temperature program was set as follows: the initial temperature of the column was 80 °C and held for 1 min, then increased to 210 °C at 8 °C/min and held for 1 min at 180 °C, then increased to 260 °C at 20 °C/min and maintained for 1 min. The flow rate was 1 ml/min. The injection temperature was 250 °C. The ion source of the mass spectrometer was set at 280 °C. Range of mass charge ratio (*m/z*) was set at 30–450.

2.2.4. NMR analysis

NMR spectra (^1H NMR and ^{13}C NMR) were recorded on a Bruker AVANCE 500 MHz spectrometer (Rheinstetten, Germany) using tetra-methyl silane (TMS) as internal standard and D_2O as the solvents at 25 °C.

2.3. Sulfation of CSPS-1

2.3.1. Preparation of sulfating reagent

The sulfation reagent, complex of CSA and Pyr was prepared by slowly adding CSA into Pyr (25 ml) in a three-necked flask, with continuous stirring and cooling in an ice bath by keeping the temperature at 4 °C. The ratio of CSA to Pyr is referred to Table 1.

2.3.2. Sulfation reaction

The sulfation reaction of CSPS-1 was done by CSA-Pyr method as described by Wang, Li, and Chen (2009). CSPS-1 (100 mg) was suspended in 10 ml anhydrous dimethyl formamide (DMF), and then the sulfation reagent was added. The mixture was treated with different reaction temperatures and times as shown in Table 1. After the sulfation, the reaction mixture was cooled to room temperature, neutralized with 2.5 M NaOH and precipitated with 95% ethanol. The sediment was re-dissolved with water and dialyzed against tap water for 48 h and distilled water for 24 h to remove pyridine and potential degradation products. As results, nine sulfated polysaccharides named CSPS-1-S_1, CSPS-1-S_2, CSPS-1-S_3, CSPS-1-S_4, CSPS-1-S_5, CSPS-1-S_6, CSPS-1-S_7, CSPS-1-S_8 and CSPS-1-S_9 were

Table 1
GC–MS data of methylated sugar residues of CSPS-1.

Methylated sugar residue	Retention time (min)	Molar ratio	Mode of linkage
2,3,4,6-Tetramethyl-glucose	15.768	7.53	Glc-(1→
2,3,6-Trimethyl-glucose	17.107	38.58	→4)-Glc-(1→
2,3-Dimethyl-glucose	18.614	8.32	→4,6)-Glc-(1→

obtained after lyophilization. The weight yield of sulfated polysaccharide was calculated according to the formula:

$$\text{Yield}\,(\%) = 100 \times \frac{W_1}{W_0}$$

where W_1 is the weight of sulfated derivatives and W_0 is the weight of CSPS-1.

The sulfate content of the polysaccharides was determined by the $BaCl_2$ gelatin method using K_2SO_4 as a standard after hydrolysis of the polysaccharides with 0.5 M HCl (Dodgson & Price, 1962). The degrees of sulfate substitution (DSS) were calculated according to the equation:

$$\text{DSS} = \frac{1.62 \times S\%}{(32 - 1.02 \times S\%)}$$

2.4. Assay of inhibitory activity in vitro on BGC-823 cell proliferation

The inhibition rates of CSPS-1 and its derivatives on the growth of BGC-823 cells were determined by MTT-based colorimetric method (Mosmann, 1983). Briefly, BGC-823 cells were suspended in RPMI-1640 medium supplemented with 10% FBS, 100 unit/ml of penicillin and 100 μg/ml of streptomycin at a density of 1×10^5 cells/ml. The cell suspension was pipetted into the wells (50 μl/well) of a 96-well plate and inoculated at 37 °C in a humidified 5% CO_2 incubator (Sanyo Electric Co., Ltd, Osaka, Japan) for 24 h. Then, 50 μl of test sample with different concentrations (0, 50, 100, 200 and 400 μg/ml in fresh growth medium) was added into each well separately. After 72 h incubation, MTT reagent (5.0 mg/ml) was added to each well (10 μl/well), and the plate was further incubated for 4 h in the incubator. Then, 100 μl of 10% SDS in 0.01 M HCl was added into each well and kept overnight for the dissolution of formazan crystals. Finally, the Abs at 570 nm of each well was read by an ELISA plate reader (TECAN Infinite F200, Switzerland). The inhibitory rate was calculated according to the formula below:

$$\text{Inhibition rate}\,(\%) = \left(\frac{1 - \text{Abs}_1}{\text{Abs}_0}\right) \times 100$$

where Abs_1 and Abs_0 are the Abs of test sample and control, respectively.

2.5. Statistical analysis

The data were expressed as mean ± standard deviation (SD) and analyzed by using SPSS for Windows Version 13.0 (SPSS, Chicago, IL). Significance at $P < 0.05$ was determined by one-way analysis of variance (ANOVA) followed by the Duncan's multiple-range tests.

3. Results and discussion

3.1. Structural characterization of CSPS-1

The glycosidic linkage locations of polysaccharides may be preliminarily determined by consumption of $NaIO_4$ and production of formic acid in periodic acid oxidation (Qiao et al., 2010). In the periodic acid oxidation of CSPS-1, 1 mol of sugar residue consumed 0.970 mol of $NaIO_4$ and produced 0.161 mol of formic acid. After 48 h of periodate treatment, the consumed amount of $NaIO_4$ was about two times more than the amount of formic acid produced, indicating that CSPS-1 contained linkages which could produce formic acid such as (1 → 6) linkage, but also contained some amount of linkage types which only could consume $NaIO_4$ such as (1 → 4) linkage (Luo, Sun, Wu, & Yang, 2012). In order to obtain more information about the glycosidic linkage location, the product of periodic acid oxidation was degraded (named Smith degradation) and analyzed by GC (Qiao et al., 2010). Fig. 1 shows the GC chromatogram of CSPS-1 derivative from periodic acid oxidation and Smith degradation. The predominant presence of glycerol and erythritol revealed that glucose residues of CSPS-1 were linked mainly by 1 → 4 glycosidic bonds.

To further determine the linkages of monosaccharides in CSPS-1, CSPS-1 was methylated, hydrolyzed and converted into the corresponding alditol acetates for GC–MS analysis. The FT-IR spectra of CSPS-1 and methylated CSPS-1 are shown in Fig. 2a. The absorption band at about 3400 cm^{-1} for O—H stretching vibrations, existing prior to methylation, disappeared. In addition, the absorption peak at about 3000–2800 cm^{-1} for the C—H stretching vibrations in methylated CSPS-1 was stronger than it was prior to methylation. The results suggested that CSPS-1 had been completely methylated (Ciucanu & Kerek, 1984). According to the ion fragment peaks (Fig. 2b) and peak areas (Table 1), three sugar residues could be identified as 2,3,4,6-tetramethyl-glucose (non-reducing terminal), 2,3,6-trimethyl-glucose (1,4-linked glucose) and 2,3-dimethyl-glucose (1,4,6-linked glucose) in molar ratios of 7.53:38.55:8.32. These molar ratios agreed with the overall monosaccharide compositions described previously (Jiang et al., 2011). The combined results from the analysis of periodate oxidation, Smith degradation and GC–MS indicated that the backbone chain of CSPS-1 was composed of glucose linked by 1 → 4 glycosidic bonds, and the branch chains were attached to backbone chain by 1 → 6 glycosidic bonds.

Fig. 4 presents the ^{13}C NMR spectrum of CSPS-1. The signal at 99.78 ppm suggested that the sugar residues of CSPS-1 were linked by α-configuration glycosidic bond. Peaks at 77–80 ppm were produced by C-4, and signals at 70–77 ppm were assigned to un-substituted C-2, C-3 and C-4. The signal at 69.34 ppm was created by substituted C-6. The results from NMR are consistent with those obtained in GC–MS analysis.

3.2. Sulfation of CSPS-1

There are several methods to sulfate polysaccharides, including sulfuric acid, sulfur trioxide-pyridine, CSA-Pyr and sulfur trioxide-dimethylacetamide (Lu, Wang, Hu, Huang, & Wang, 2008). Among them, the CSA-Pyr method has many advantages, such as high yield and convenient isolation of product (Baba, Snoeck, Pauwels, & De Clercq, 1988). In use of this method, the ratio of CSA to Pyr, the reaction temperature and reaction time are generally considered to be the most important factors (Yang, Jia, Shang, Mei, & Zhao, 2001). Therefore, the ratio of CSA to Pyr, reaction temperature and reaction time were optimized in the present study by using an orthogonal test design of $L_9(3^4)$. As shown in Table 2, CSPS-1-S_5 had the highest DSS of 1.02, followed by CSPS-1-S_2 with DSS 0.88, and CSPS-1-S_1 with DSS of 0.3. Compared to CSPS-1 (Jiang et al., 2011), the yields of the sulfated derivatives were relatively lower,

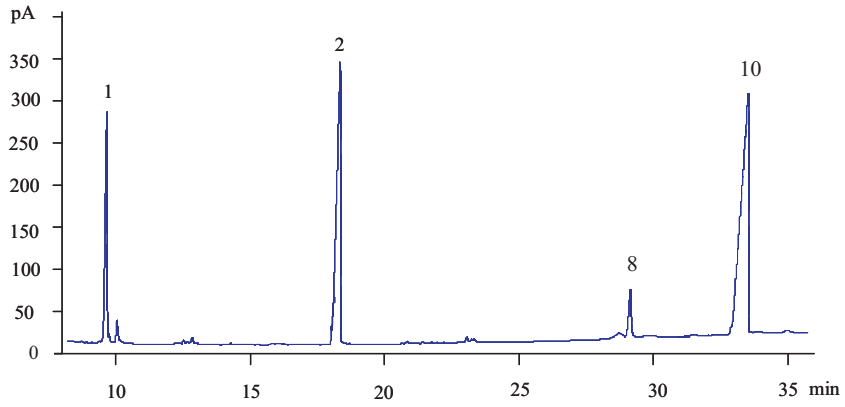

Fig. 1. GC chromatogram of derivative from CSPS-1 after periodic acid oxidation and Smith degradation (1—glycerol; 2—erythritol; 8—glucose; 10—inositol).

ranging from 50.85% to 72.19%. The result is in accordance with the previous report that the yield of some polysaccharides decreased after sulfation (Lu et al., 2008).

Analyses on the orthogonal array design showed that the extent of variable impact on DSS followed the order: variable C (reaction time) < variable B (reaction temperature) < variable A (molar ratio of CSA to Pyr). The results indicate that the molar ratio of CSA to Pyr is the major factor affecting DSS. In addition, the DSS of product increased rapidly with increase of the molar ratio of CSA to Pyr, but when the molar ratio of CSA to Pyr was higher than 1:4, the

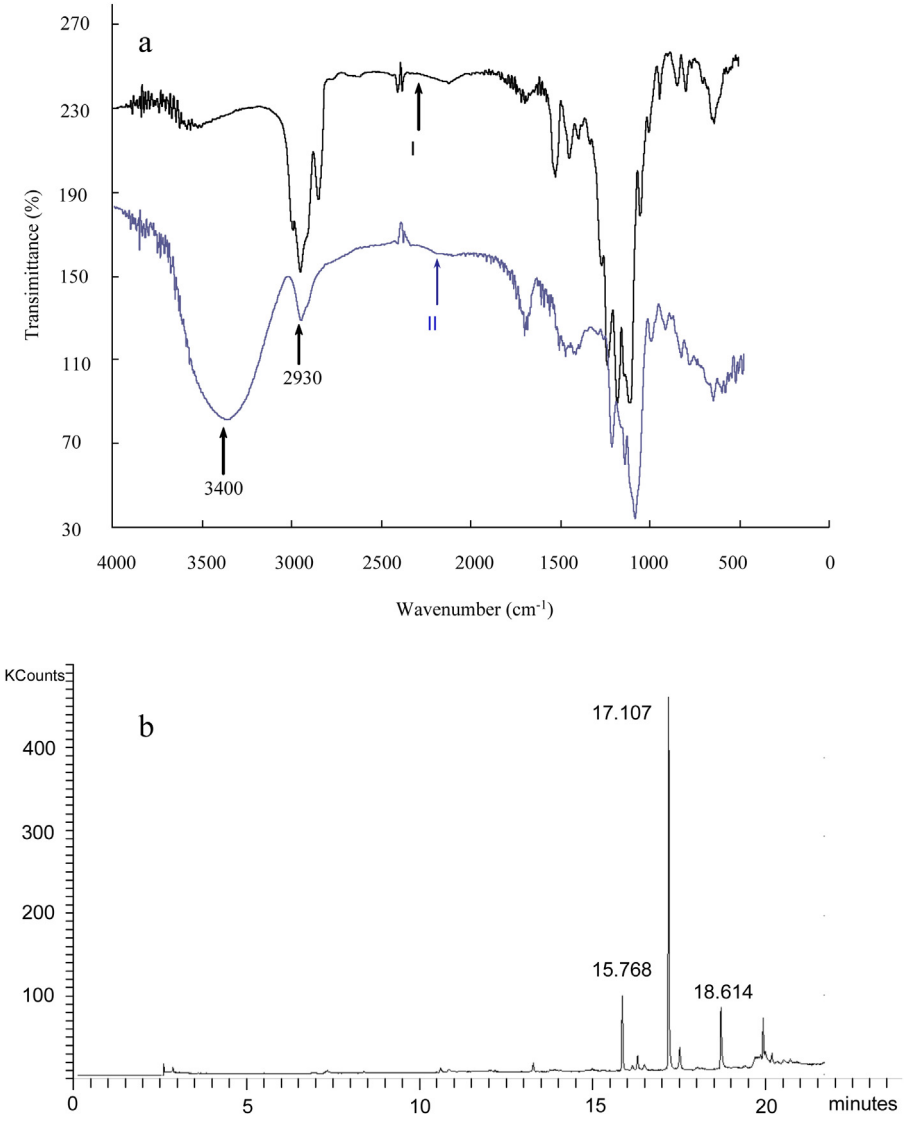

Fig. 2. FT-IR spectrum (a) and GC–MS chromatogram (b) of CSPS-1 after methylation reaction (I—methylated CSPS-1; II—CSPS-1).

Table 2
The DSS and yield of sulfated CSPS-1.

CSPS-1-S	Sulfated conditions			DSS and yield of derivatives	
	(A) Temperature (°C)	(B) CSA: Pyr	(C) Time (h)	DSS	Yield (%)
CSPS-1-S_1	1 (50)	1 (1:6)	1 (2)	0.30 ± 0.04	68.20 ± 1.26
CSPS-1-S_2	1	2 (1:4)	2 (3)	0.88 ± 0.06	67.11 ± 1.41
CSPS-1-S_3	1	3 (1:2)	3 (4)	0.53 ± 0.07	60.71 ± 1.05
CSPS-1-S_4	2 (65)	1	3	0.74 ± 0.07	70.61 ± 2.18
CSPS-1-S_5	2	2	1	1.02 ± 0.08	72.19 ± 1.91
CSPS-1-S_6	2	3	2	0.85 ± 0.01	64.73 ± 1.13
CSPS-1-S_7	3 (80)	1	2	0.41 ± 0.08	60.19 ± 2.43
CSPS-1-S_8	3	2	3	0.76 ± 0.11	50.85 ± 1.64
CSPS-1-S_9	3	3	1	0.79 ± 0.07	52.10 ± 1.71

DSS of product decreased. Furthermore, the effects of the sulfation variables on the yields were also examined. The extent of variable impact on the yield followed the order: variable A < variable C < variable B. Notably, the reaction temperature was the major factor affecting the yield of polysaccharide derivatives. With the increase of reaction temperature from 50 to 65 °C, the yield of sulfated product increased rapidly. However, it decreased when the temperature was up to 80 °C. This might be ascribed to the possibility that higher temperature accelerated chain cleavage and further degradation reactions.

The analysis of the orthogonal array design results indicates that the reaction temperature and molar ratio of CSA to Pyr are the two major factors affecting the DSS and yield of derivatives. The increase of reaction temperature and the molar ratio of CSA to Pyr could contribute to high DSS and yield of products. However, excessive reaction temperature and/or molar ratio of CSA to Pyr would cause decrease of the DSS and yield. Therefore, sulfation reaction should be in a relatively mild condition. Taking the DSS or yield as main index, reaction time exerted less effect. The DSS of product reached 0.70 at a reaction time of 1 h, after that the DSS remained unchanged with the increase of reaction time. The result is consistent with the finding that about 85% of the possible substitution occurred within the first hour during the sulfation of Chinese lacquer polysaccharides (Yang, Du, Huang, Wan, & Li, 2002).

The FT-IR spectra of CSPS-1 and CSPS-1-S are shown in Fig. 3. Compared with CSPS-1, two new characteristic absorption bands (one at near 1210 cm^{-1} describing an asymmetrical S=O and another at near 813 cm^{-1} representing a symmetrical C—O—S vibration) appeared in the FT-IR spectra of CSPS-1-S_1, CSPS-1-S_3 and CSPS-1-S_9, confirming sulfate modification of CSPS-1 (Zhang, Zhang, Zhou, Chen, & Zeng, 2000). In addition, CSPS-1-S_9 showed significant absorption bands at 1210 cm^{-1} or 813 cm^{-1}, indicating higher DSS in CSPS-1-S_9. The relative intensities of these absorptions coincide with the measured degrees of substitution.

The position of sulfation occurred to polysaccharides can be further determined by NMR technologies (Li et al., 2014). The ^1H and ^{13}C NMR spectra of CSPS-1 and CSPS-1-S are shown in Fig. 4. Some new signals between 3.5 and 4.7 ppm were observed in the ^1H NMR spectrum of CSPS-1-S (Fig. 4B) compared to that of CSPS-1 (Fig. 4A), indicating the successful sulfation of CSPS-1. The carbon signal at about 60 ppm was obviously weakened while the signal at about 67 ppm was strongly enhanced (Fig. 4C and D), indicating that some OH groups at C-6 of CSPS-1 were substituted by sulfate groups. The results indicated that sulfation occurred at least partially at C-6 of CSPS-1.

3.3. Antitumor activities of CSPS-1 and CSPS-1-S

Previous studies have confirmed that the introduction of sulfate groups to polysaccharides may induce changes in physicochemical properties and chain conformation, and sulfation of polysaccharides is beneficial to the enhancement of antitumor activity (Lin et al., 2004; Tao, Zhang, & Cheung, 2006). Accordingly, the growth inhibitory effects of CSPS-1 and its sulfated derivatives against

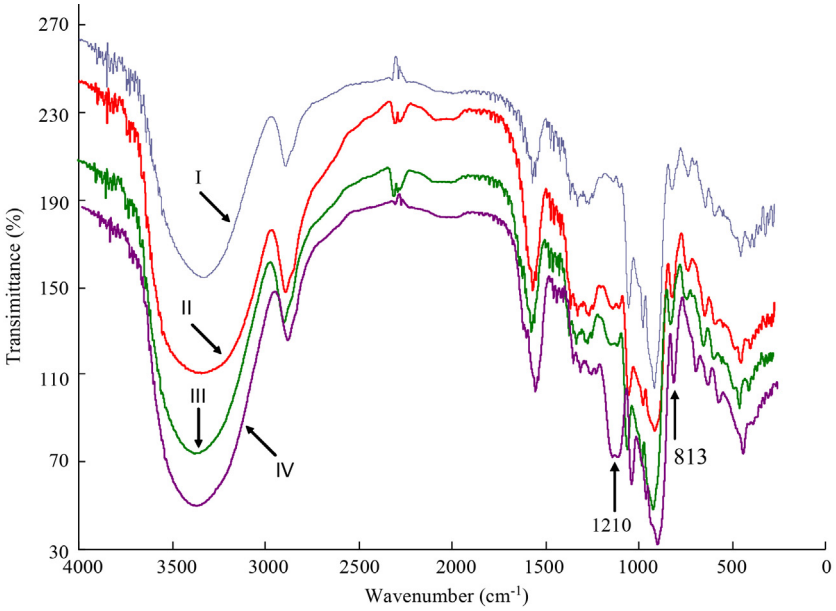

Fig. 3. FT-IR spectra of sulfated CSPS-1 (I—CSPS-1; II—CSPS-1-S_1; III—CSPS-1-S_3; IV—CSPS-1-S_9).

Table 3
Inhibitory effects *in vitro* of CSPS-1-S at different concentrations against human gastric cancer BGC-823 cells at 72 h.

Groups	Concentration (μg/ml)			
	50	100	200	400
CSPS-1	20.27 ± 1.50^{d}	28.59 ± 3.92^{d}	34.22 ± 1.50^{d}	41.22 ± 1.35^{e}
CSPS-1-S_1	33.39 ± 4.47^{c}	48.22 ± 4.42^{c}	65.43 ± 1.56^{b}	73.68 ± 1.02^{b}
CSPS-1-S_2	51.36 ± 4.35^{b}	70.91 ± 1.18^{b}	77.54 ± 2.88^{a}	78.06 ± 1.94^{b}
CSPS-1-S_3	45.62 ± 4.08^{b}	54.67 ± 6.04^{c}	56.90 ± 3.66^{c}	74.48 ± 1.18^{b}
CSPS-1-S_4	72.95 ± 5.19^{a}	72.52 ± 6.06^{b}	79.36 ± 8.37^{a}	86.90 ± 2.33^{a}
CSPS-1-S_5	69.50 ± 1.55^{a}	80.60 ± 0.77^{a}	84.17 ± 2.01^{a}	89.44 ± 6.54^{a}
CSPS-1-S_6	48.18 ± 2.48^{b}	51.22 ± 3.24^{c}	64.34 ± 3.96^{b}	73.20 ± 2.86^{bc}
CSPS-1-S_7	45.17 ± 4.76^{b}	47.17 ± 6.60^{c}	53.86 ± 3.41^{c}	68.62 ± 1.72^{c}
CSPS-1-S_8	20.15 ± 1.56^{d}	24.36 ± 1.60^{d}	38.29 ± 1.77^{d}	59.51 ± 2.41^{d}
CSPS-1-S_9	19.48 ± 0.35^{d}	26.59 ± 2.64^{d}	40.43 ± 3.58^{d}	59.13 ± 1.17^{d}

Values are presented as means ± standard deviation (SD) of three independent experiments. Different alphabets (a–d) in superscript for each treatment (50, 100, 200 or 400 μg/ml) donate significant difference ($P < 0.05$).

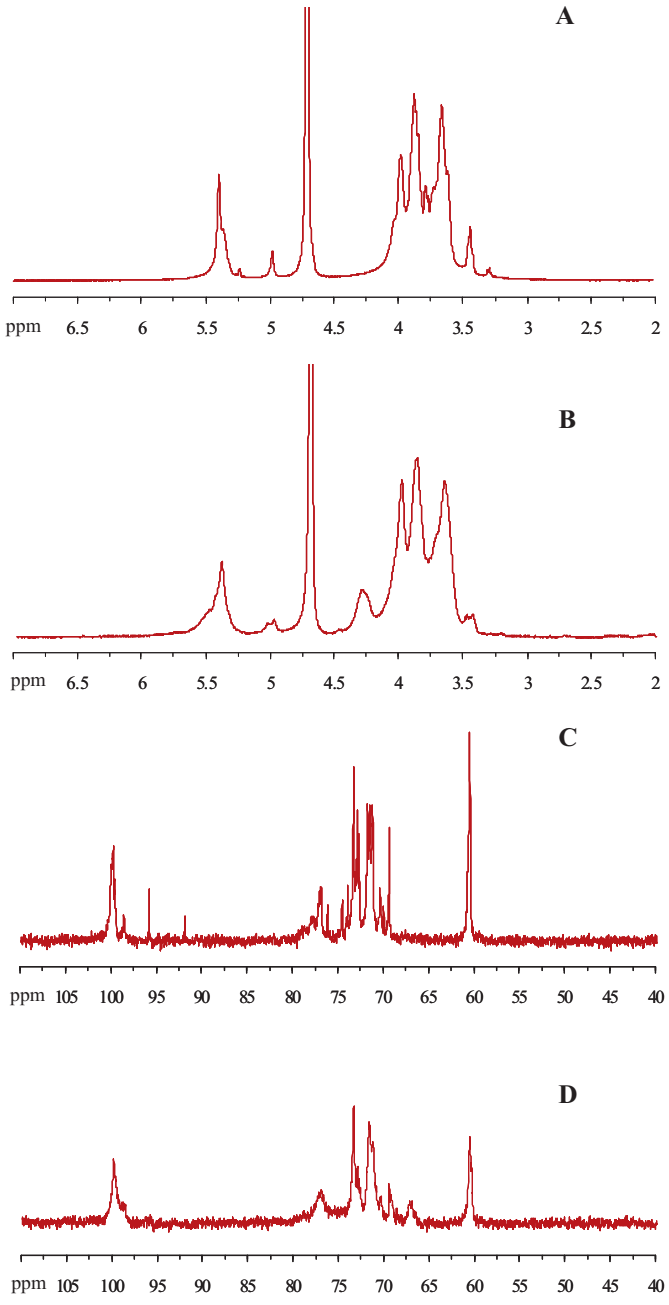

Fig. 4. ^{1}H NMR spectra for CSPS-1 (A) and CSPS-1-S (B) and ^{13}C NMR spectra for CSPS-1 (C) and CSPS-1-S (D).

BGC-823 cells *in vitro* were examined. As shown in Table 3, CSPS-1 and its sulfated derivatives exhibited dose-dependent activity within the concentration range of 50–400 μg/ml, and the inhibitory effect of the polysaccharide derivatives increased significantly ($P < 0.05$) with the increase of sample concentration. Compared to CSPS-1, the sulfated derivatives showed much higher inhibitory activity against BGC-823 cells, indicating that the antitumor activity of CSPS-1 could be enhanced by sulfation.

It had been demonstrated that a moderate DSS was necessary for a high antitumor activity *in vitro* (Chen et al., 2011). We also found that when the DSS was within the range of 0.41–1.02, the derivatives exhibited relatively stronger antitumor activity *in vitro*. This might be due to the fact that the sulfated polysaccharides with high DSS are often accompanied with low molecular weight and damaged structure of polysaccharides resulted from longer reaction time in the strong acidic environment (Chen et al., 2011; Yang, Du, Wen, Li, & Hu, 2003). Therefore, the DSS of polysaccharides must be within the optimal ranges. According to DSS, yield and antitumor activity, the optimal sulfation conditions for CSPS-1 were determined to be reaction temperature of 65 °C, reaction time of 2 h and CSA-Pyr ratio of 1:4.

The activity of a polysaccharide may be positively related to its intestinal absorption efficiency, which is influenced by its molecular weight and water-solubility (Zhang et al., 2014). It has been reported that the absorption efficiency of polysaccharides increased with the decrease of molecular weight (Balogh et al., 2008; Zeng, Qin, Wang, Chi, & Li, 2008). In the present study, the improvement of antiproliferative activity might be due to the decreased molecular weight of CSPS-1 after sulfation. Many studies have suggested that the polysaccharides exert their antitumor effects by inducing apoptosis or activating immune responses in the host (Wang, Sun, Wu, Yang, & Tan, 2014). However, the mechanisms of antitumor action of the sulfated CSPS remain unclear. Studies on antitumor mechanism of the sulfated CSPS are in progress.

4. Conclusions

Through characterization, we have demonstrated that the backbone chain of CSPS-1 is composed of glucose linked by α-(1 → 4) glycosidic bonds, and the branch chains are attached to backbone chain by 1 → 6 glycosidic bonds. CSPS-1 was successfully sulfated by the CSA-Pyr method to afford nine sulfated polysaccharides. Furthermore, the optimal sulfation conditions for CSPS-1 were determined to be reaction temperature of 65 °C, reaction time of 2 h and CSA-Pyr ratio of 1:4. Structural analysis revealed that sulfation occurred at position of C-6 of glycosyl residue. In addition, the modified CSPS-1 had significantly enhanced inhibitory activity *in vitro* on BGC-823 cell proliferation in comparison with non-sulfated CSPS-1.

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