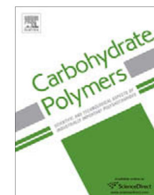
Carbohydrate Polymers 77 (2009) 370–375

Contents lists available at ScienceDirect

Carbohydrate Polymers

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



Sulfation of a polysaccharide obtained from *Phellinus ribis* and potential biological activities of the sulfated derivatives

Yuhong Liu [a,b,c], Chunhui Liu [a,b], Haining Tan [b], Ting Zhao [a], Jichao Cao [a], Fengshan Wang [a,b,*,1]

[a] *Institute of Biochemical and Biotechnological Drugs, School of Pharmaceutical Sciences, Shandong University, No. 44 West Wenhua Road, Jinan 250012, PR China*
[b] *National Glycoengineering Research Center, Shandong University, No. 44 West Wenhua Road, Jinan 250012, PR China*
[c] *Shandong University of Traditional Chinese Medicine, Jinan 250355, PR China*

ARTICLE INFO

Article history:
Received 21 October 2008
Received in revised form 4 December 2008
Accepted 9 January 2009
Available online 20 January 2009

Keywords:
Phellinus ribis
Polysaccharide
Sulfation
Antitumor
Anti-angiogenic activities

ABSTRACT

The paper reports the preparation, characterization and potential biological activities of a chemically sulfated polysaccharide isolated from *Phellinus ribis*. Four sulfated derivatives (PRP-SI–IV) with variable degrees of substitution were obtained by the chlorosulfonic acid method, without degradation of the polysaccharide (PRP). The sulfate groups were not regularly distributed along the polysaccharide chain with a multiple substitution pattern as determined by ^{13}C NMR. The sulfated derivatives except for PRP-SI showed significant inhibition effects on HepG2 cells in comparison with the native non-sulfated polysaccharide (PRP). All sulfated derivatives could block new angiogenic vessel formation in zebrafish assay, however, the effects were less than PRP.

© 2009 Elsevier Ltd. All rights reserved.

1. Introduction

Sulfated polysaccharides, widespread in nature, play an important role in molecular recognition, cell development and differentiation, and cell–cell interaction. A number of natural sulfated polysaccharides exhibit diverse biological activities, such as anticoagulant activity (Athukorala, Jung, Vasanthan, & Jeon, 2006; Carlucci et al., 1997), antiviral activity (Haslin, Lahaye, Pellegrini, & Chermann, 2001; Preeprame, Hayashi, Lee, Sankawa, & Hayashi, 2001), antitumor activity (Parish, Freeman, Brown, Francis, & Cowden, 1999; Wu, Chen, & Xie, 2006; Zhou et al., 2004), and so on. Activities of these polysaccharides are strictly related to the presence of polyanionic charges (Martinichen-Herrero, Carbonero, Sassaki, Gorin, & Iacomini, 2005), which has led to chemically sulfated modification of many natural polysaccharides. 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 (Chaidedgumjorn et al., 2002; Qiu, Tang, Tong, Ding, & Zuo, 2007; Shi, Nie, Chen, Liu, & Tao, 2007). Chemical modification of polysaccharides provided an opportunity to obtain new pharmacological agents with possible therapeutic uses.

Phellinus ribis has been used for treating pharyngitis and enhancing immunity as a Chinese folk medicine. In our previous work, a polysaccharide with a mean molecular weight of 8.59 kDa, named as PRP, was isolated from the fruiting bodies of *P. ribis* for the first time (Liu & Wang, 2007). It is a β-D-glucan containing a (1 → 4), (1 → 6)-linked backbone, with a single β-D-glucose at the C-3 position of (1 → 6)-linked glucosyl residue every eight residues along the main chain. PRP has an obvious immunostimulating effect, but has no antitumor activity *in vitro*. To seek new active compounds, we prepared sulfated derivatives of PRP by chemical modification, and studied the biological activities of the sulfated derivatives with different degree of substitution (DS) in this paper.

2. Experimental

2.1. Materials

P. ribis was collected from the mountain area in Jinan city, Shandong Province, China in October 2007. Chlorosulfonic acid was produced in Guoyao Institute of Chemical Engineering in Shanghai. Formamide was from Beijing Yili Co. Ltd., China. DEAE–Sepharose Fast Flow was from Pharmacia Co. (Sweden). Medium RPMI-1640 and fetal bovine serum (FBS) were purchased from Gibco-BRL, Life Technologies, Inc., USA. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphe-

* Corresponding author. Address: Institute of Biochemical and Biotechnological Drugs, School of Pharmaceutical Sciences, Shandong University, No. 44 West Wenhua Road, Jinan 250012, PR China. Tel.: +86 531 88382589; fax: +86 531 88382548.
E-mail addresses: liuyuhongwu@yahoo.com.cn (Y. Liu), liuchunhui@sdu.edu.cn (C. Liu), tinytan@yahoo.cn (H. Tan), zhaoting2110000@yahoo.com.cn (T. Zhao), caojichao@sdu.edu.cn (J. Cao), fswang@sdu.edu.cn, wangfengshansdu@hotmail.com (F. Wang).
[1] Tel.: +86 531 88382589; fax: +86 531 88382548.

0144-8617/$ - see front matter © 2009 Elsevier Ltd. All rights reserved.
doi:10.1016/j.carbpol.2009.01.008

nyltetrazolium bromide (MTT) was purchased from Sigma Chemicals Co., USA. 5-Fluorouracil (5-Fu) was from Shanghai Xundonghaipu Pharmaceutical Co. Ltd., China. All other reagents were of analytical grade made in China.

2.2. General methods

Optical rotation was measured at 20 °C with a WZZ-1 polarimeter. UV–vis absorption spectra were recorded with a Unico™ UV-2102PC spectrophotometer. FT-IR spectra (KBr pellets) were recorded on a Nicolet Nexus 470 FT-IR spectrophotometer. ^{13}C NMR spectra were recorded at 40 °C with a Bruker Avance 600 MHz spectrometer (Germany), and the chemical shifts were expressed in ppm relative to the resonance of internal standard DSS. Elemental analysis (C, H, S) was conducted on a Perkin-Elmer 2400 instrument. Protein was measured by the Folin-phenol method using bovine serum albumin as standard.

2.3. Preparation of the sulfated derivatives

PRP was obtained by the method reported previously (Liu & Wang, 2007). Chemical sulfation of PRP was carried out using the chlorosulfonic acid method (Yoshida, Yasuda, Mimura, & Kaneko, 1995). To obtain sulfated derivatives with variable DS, four kinds of sulfating reagent (the ratio of chlorosulfonic acid to formamide of 1:8, 1:4, 1:2 and 1:1) were prepared using dry formamide and chlorosulfonic acid according to the reported method (Lu, Wang, Hu, Huang, & Wang, 2008). In brief, PRP sample (200 mg) was suspended in anhydrous formamide (2 ml) at room temperature with stirring for 30 min, and the sulfating reagents (2 ml) were added dropwise. The mixture was maintained at 30 °C for 6 h with continuous stirring. After the reaction was finished, the mixture was cooled, neutralized with 2.5 M NaOH solution, and treated by adding ethanol. The resulting precipitate was redissolved in water and dialyzed against distilled water. The retained nondialysate was treated with ethanol again. The precipitate was dissolved in water, applied to DEAE–Sepharose Fast Flow column (2.6 × 20 cm), and eluted with water and NaCl solution with a concentration gradient of 0–2.0 M. Based on the colorimetric test for total carbohydrate by phenol–sulfuric acid method, the main fraction was collected, dialyzed and lyophilized to give sulfated PRP (PRP-S).

2.4. Homogeneity and molecular weight determination

The homogeneity and molecular weight of the sulfated derivatives were determined by high-performance gel-permeation chromatography (HPGPC) on a Waters 515 instrument equipped with an Ultrahydrogel 250 column (7.8 × 300 mm) and a Waters 2410 Refractive Index Detector (RID). Twenty microliters of sample solution (0.5% PRP-S solution) was injected in each run, with 0.05 M Na_2SO_4 as the mobile phase at a flow rate of 0.8 ml/min. The HPGPC system was precalibrated with T-series Dextran standards (T-10, T-20, T-40, T-70, T-110 and T-500).

2.5. Growth inhibition assay on HepG2 cells

The inhibition effects of the sulfated derivatives and PRP on the growth of HepG2 cells were evaluated *in vitro* by colorimetric MTT assay as described previously (Mossmann, 1983). Cells were seeded in a 96-well plate with 190 μl per well at a density of 2.5×10^4 cells/ml in RPMI-1640 medium and incubated for 24 h at 37 °C in a humidified 5% CO_2 incubator. Then the sample (10 μl, concentration range: 200–10,000 μg/ml) and 5-Fu (10 μl, 500 μg/ml) were added. After incubation for another 48 h, 10 μl of MTT (5 mg/ml) was added into each well and incubated for another 4 h. Thereafter, the supernatant was removed carefully, and 150 μl of DMSO was added to each well. The absorbance at 570 nm was measured with an ELISA reader (Bio-Rad Model 6800, USA). The inhibition ratio (IR) was calculated according to the formula below:

IR (%) = (1 − Absorbance of experimental group/Absorbance of blank control group) × 100%.

2.6. Angiogenesis inhibition assay with live fluorescent zebrafish

2.6.1. Embryo handling

GFP transgenic zebrafish embryos, from Institute of Biology, Shandong Academy of Sciences, were maintained at 28 °C on a 14 h light/10 h dark cycle. All embryos were generated by natural pair-wise mating and staged according to Kimmel, Ballard, Kimmel, Ullmann, and Schilling (1995). Four to five pairs were set up for each mating, on average 100–150 embryos per pair were generated. Embryos were maintained in embryo water (5 g of Instant Ocean Salt in 25 L of distilled water, pH 7.4) at 28 °C for 20 h until the 21 somite stage before sorting for viability using both morphology and developmental stage as criteria. Healthy embryos were dechorionated by enzymatic digestion with 1 mg/ml protease for 5 min at room temperature. The embryos were then washed five times in embryo water. Because the embryos receive nourishment from an attached yolk ball for the duration of the experiment, no additional maintenance was required.

2.6.2. Treatment with samples

Samples dissolved in embryo water were transferred in a 96-well plate with 200 μl per well. One healthy embryo at 20 h post-fertilization was added to each well. The 96-well plate was maintained at 28 °C on a 14 h light/10 h dark cycle. For each treatment, 12 embryos were used.

2.6.3. Intersegmental vessel counting

After incubation for another 52 h, zebrafish were anesthetized by tricaine and the intact intersegmental vessels (ISV) were counted using a COIC XSZ-H fluorescence microscope (Chongqing Photoelectric Instrument Co. Ltd., China.). The inhibition ratio (IR) was calculated according to the formula below:

IR (%) = (1 − Amount of experimental group/Amount of blank control group) × 100%.

2.7. Statistical analysis

The data obtained were expressed as mean ± *SD* and analyzed statistically by ANOVA method. Significance of any differences between groups was evaluated using the Student's *t*-test.

3. Results and discussion

3.1. Characterization of the sulfated derivatives

It was reported that controlling the reagent amount was better than controlling the reaction temperature to get sulfated polysaccharide derivatives with different DS (Vogl, Paper, & Franz, 2000). Four sulfated derivatives of PRP named PRP-SI, PRP-SII, PRP-SIII and PRP-SIV were obtained by varying the ratio of chlorosulfonic acid to formamide in the sulfating reagent (Table 1). They all showed a symmetrical peak on HPGCP, and the average molecular weights of PRP-SI, PRP-SII, PRP-SIII and PRP-SIV were determined to be 14.61, 20.54, 21.42 and 22.43 kDa, respectively, in reference to standard T-Dextran, indicating that almost no degradation occurred in the sulfation reaction process. The DS was calculated according to the ratio of carbon to sulfur by element analysis (DS means the amount of mole of sulfur per mole of glycose residue) (Yoshida et al., 1995), shown in Table 1. The DS showed a lin-

Table 1
Characterization of the sulfated PRP.

PRP-S[a]	CSA:FA[b]	Yield (mg)	$\bar{M}_w$ (kDa)	α_D^{20} (°)	Elemental analysis (%)			DS
					C	H	S	
PRP-SI	1:8	196	14.61	+9.87	29.32	4.73	8.08	0.62
PRP-SII	1:4	248	20.54	+2.63	18.89	3.33	12.74	1.52
PRP-SIII	1:2	262	21.42	−2.46	16.29	2.48	13.05	1.80
PRP-SIV	1:1	286	22.43	−3.42	16.07	2.49	14.46	2.02

[a] From the parent PRP 200 mg.
[b] The ratio of chlorosulfonic acid to formamide in sulfating reagent.

ear increase with the ratio of chlorosulfonic acid to formamide under constant reaction conditions.

The molecular weight of sulfate polysaccharide is an important parameter influencing bioactivity. In the sulfation reaction some polysaccharide degradation usually occurs (Han, Yao, Yang, Liu, & Gao, 2005; Qiu et al., 2007; Yang, Du, Yan, Li, & Hu, 2003). Research indicates that reaction temperature was a major factor in the degradation of polysaccharides and an appropriate solvent could prevent degradation (Yang et al., 2003). In our experiment four sulfated derivatives without degradation were prepared successfully, which was maybe because formamide was used as the solvent and a moderate reaction temperature (30 °C) was used during the sulfation reaction.

Sulfated PRP had a negative response to the Lowry test and no absorption at 280 nm or 260 nm in the UV spectrum, indicating the absence of protein and nucleic acid. By comparison with PRP, two characteristic absorption bands appeared in the FT-IR spectra of the sulfated derivatives (Fig. 1), one at near 1258 cm^{-1} describing an asymmetrical S=O stretching vibration and the other at near 810 cm^{-1} representing a symmetrical C–O–S vibration associated with a $C–O–SO_3$ group, indicating PRP-SI, PRP-SII, PRP-SIII and PRP-SIV were successfully sulfated (Mähner, Lechner, & Nordmeier, 2001).

The sulfated position in the polysaccharide was usually determined by NMR spectrum or methylation analysis. However, it has been reported that the sulfate group would be removed under alkaline condition during methylation analysis (Kovensky, Covián, & Cirelli, 1990). Therefore, structural characterizations of the sulfated derivatives were performed by NMR spectrum. The ^{13}C NMR spectra of the sulfated PRP were presented in Fig. 2. Comparing the signals of PRP-S with those of PRP assigned previously (Liu & Wang, 2007), 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 (Gamazade et al., 1997). The new peaks at near δ 70 in ^{13}C NMR spectra of the sulfated derivatives of PRP were assigned to the signals of the O-6 substituted carbons, suggesting sulfation of O-6. Two C-6 speaks attributed to terminal and $(1 \rightarrow 4)$-glucosyl residues of PRP still remained at 63 ppm and 65 ppm for the heterogeneously sulfated products, suggesting that the primary OH groups on the internal side of the helix were not sulfated. It is known that the signal of C-1 splits if hydroxyl group on C-2 is functionalized (Richter & Wagenknecht, 2003; Yoshida et al., 1995). A multiple split of signals for C-1 appeared in the anomeric region. Therefore, all can be assigned to C-1 either without a sulfate group on C-2 (low-field signals) or for C-1 with a sulfate group on C-2 (high-field signals). New peaks at 79–85 ppm and overlap of signals at 73–79 ppm mean sulfation of other positions occurred besides C-6 and C-2. From PRP-SI to PRP-SIV the overlap of signals was getting more severe as the electronic environment of the carbons became complex for the substitution of sulfate, meaning increase of DS, which was in agreement with the data calculated from the elemental analysis. As the consequence of a heterogeneous reaction, the sulfate groups were distributed unevenly.

3.2. Growth inhibition of PRP-S on HepG2 cells

It was reported that chemical sulfation of polysaccharides might lead to antitumor activity (Du et al., 2004; Lin, Zhang, Chen, & Jin, 2004; Williams et al., 1991). In the present study, the growth inhibitory effects of PRP-S against human hepatoma cell line HepG2 *in vitro* were first examined. The results, shown in Table 2, indicated that PRP had no obvious influence on HepG2 cells, but PRP-SII, PRP-SIII and PRP-SIV exhibited much stronger inhibition effects against HepG2 cell growth than the non-sulfated PRP, suggesting the sulfate group could contribute to direct antitumor activity *in vitro*. However, it does not seem that polysaccharide

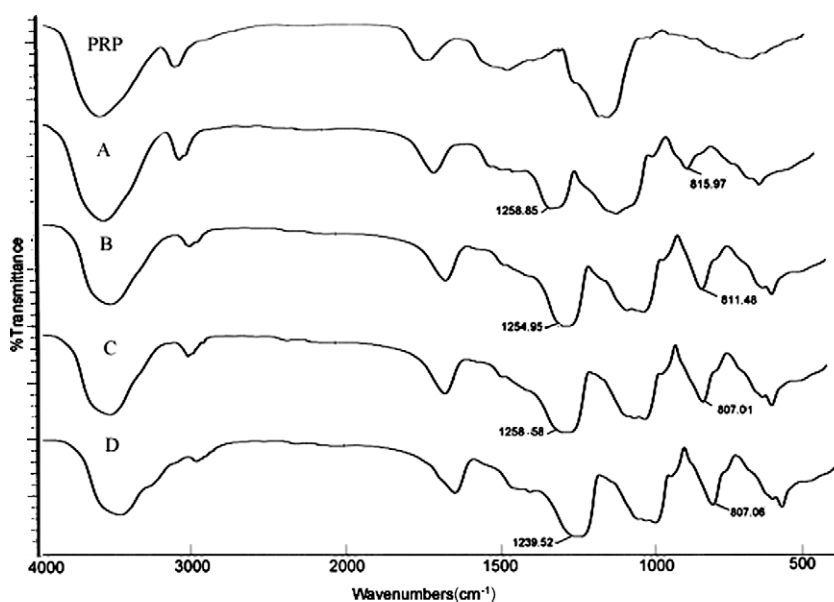


Fig. 1. FI-IR spectra of PRP and its sulfated derivatives. (A) PRPS-SI; (B) PRPS-SII; (C) PRPS-SIII and (D) PRPS-SIV.

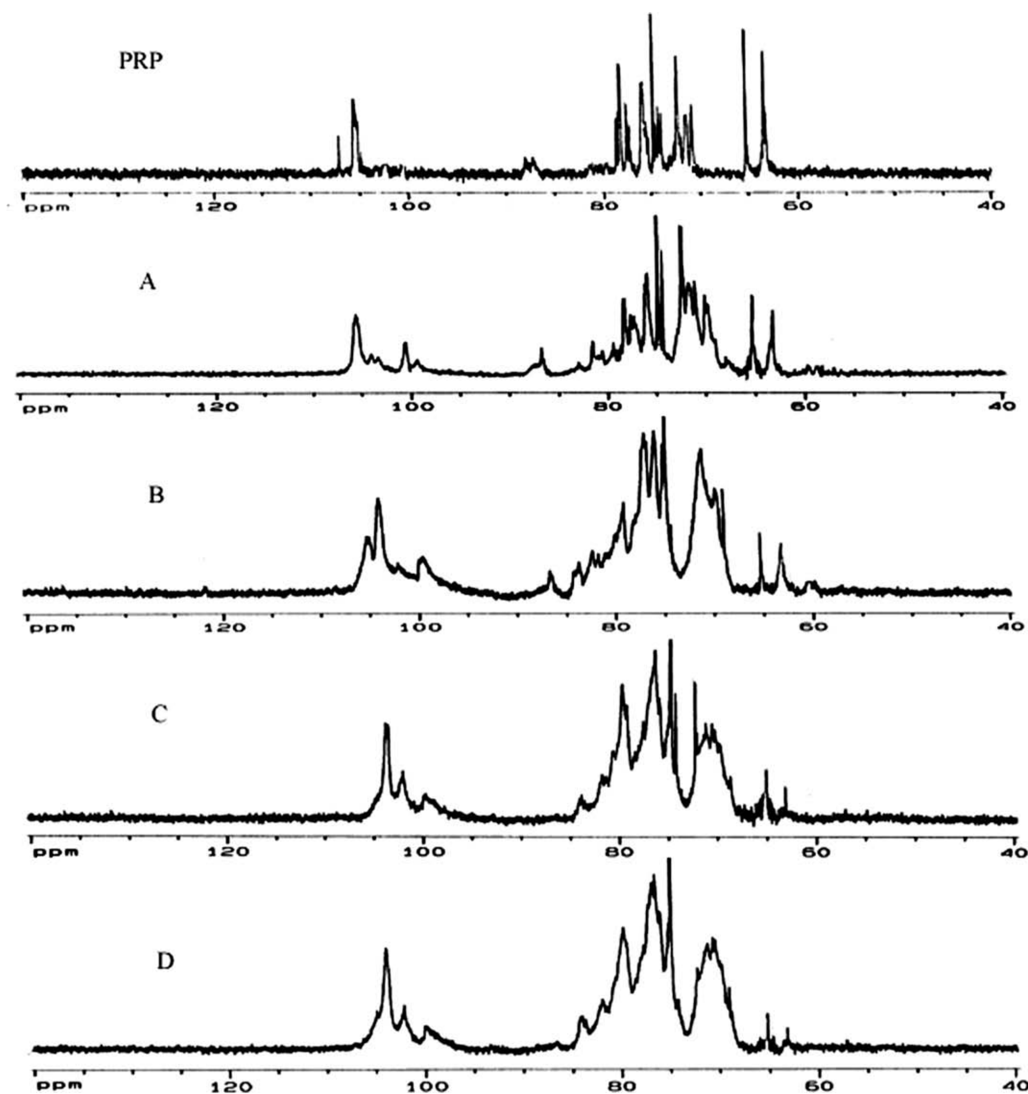


Fig. 2. ^{13}C NMR (600 MHz) spectra of PRP and its sulfated derivatives. (A) PRPS-SI; (B) PRPS-SII; (C) PRPS-SIII and (D) PRPS-SIV.

Table 2
Growth inhibition of PRP and its sulfated derivatives against HepG2 cells *in vitro*.

Group	Result	C (μg/ml)					
		10	25	50	100	250	500
PRP	*A*[a]	0.967 ± 0.003	0.951 ± 0.018	0.921 ± 0.022	0.947 ± 0.022	0.933 ± 0.019	0.949 ± 0.029
	IR (%)	−1.21	0.44	3.56	0.82	2.30	0.59
PRP-SI	*A*[a])	1.032 ± 0.030[b]	0.997 ± 0.013[b]	1.058 ± 0.022[c]	1.091 ± 0.038[c]	1.033 ± 0.032[b]	1.009 ± 0.037
	IR (%)	−11.25	−7.43	−14.07	−17.63	−11.3	−8.82
PRP-SII	*A*[a]	0.940 ± 0.009	0.921 ± 0.028	0.863 ± 0.007[b]	0.824 ± 0.006[c]	0.704 ± 0.018[c]	0.730 ± 0.019[c]
	IR (%)	0.11	2.12	6.98	11.24	24.16	21.30
PRP-SIII	*A*[a]	0.838 ± 0.020[c]	0.804 ± 0.013[c]	0.754 ± 0.024[c]	0.751 ± 0.008[c]	0.707 ± 0.012[c]	0.737 ± 0.023[c]
	IR (%)	10.91	14.58	19.80	20.09	24.87	21.67
PRP-SIV	*A*[a]	0.835 ± 0.016[b]	0.852 ± 0.006[c]	0.771 ± 0.013[c]	0.792 ± 0.019[c]	0.784 ± 0.011[c]	0.814 ± 0.015[c]
	IR (%)	11.25	9.47	18.03	15.82	16.69	13.43
Control	*A*[a]	0.955 ± 0.013	(PRP)	0.928 ± 0.031	(PRP-SI, SII)	0.941 ± 0.022	(PRP-SIII, SIV, 5-Fu)
5-Fu	*A*[a]		0.559 ± 0.016[c]				
	IR (%)		40.55				

[a] *A* means the absorption at 570 nm and the data were expressed as mean ± *SD* ($n = 6$).
[b] $P < .05$ vs. control group.
[c] $P < .01$ vs. control group.

Table 3
Inhibition effects of PRP and its sulfated derivatives on angiogenesis in zebrafish assay.

Group	Result	C (μg/ml)			
		1	10	100	1000
PRP	Amount of ISV[a]	27.86 ± 1.38	25.7 ± 1.12[b]	24.88 ± 1.25[b]	16.50 ± 1.03[b]
	IR (%)	0.51	8.16	11.16	41.07
PRP-SI	Amount of ISV[a]	27.00 ± 0.88	26.13 ± 1.05[b]	25.83 ± 1.08[b]	20.71 ± 0.97[b]
	IR (%)	3.57	6.68	7.74	26.02
PRP-SII	Amount of ISV[a]	27.50 ± 0.98	26.33 ± 0.76[b]	26.04 ± 0.71[b]	21.86 ± 0.74[b]
	IR (%)	1.79	5.95	7.02	21.94
PRP-SIII	Amount of ISV[a]	26.5 ± 0.78[b]	26.43 ± 1.13[b]	25.32 ± 0.87[b]	20.35 ± 1.18[b]
	IR (%)	5.36	5.61	9.57	27.32
PRP-SIV	Amount of ISV[a]	27.33 ± 1.23	26.57 ± 1.19[b]	26.00 ± 1.03[b]	24.00 ± 0.99[b]
	IR (%)	2.39	5.10	7.14	14.29
Control	Amount of ISV[a]	28.00 ± 0.88			

[a] ISV means the intact intersegmental vessels and the data were expressed as mean ± *SD* ($n = 12$).
[b] $P < .05$ vs. control group.

with higher sulfate content exhibits stronger antitumor activity. In the experiment, PRP-SIII with DS of 1.80 showed the highest activity with inhibition ratio of 24.87% at 250 μg/ml and the effect was in a dose-dependent manner at the concentration ranging from 10 to 250 μg/ml, suggesting a moderate DS of the sulfated derivatives was necessary for a high antitumor activity *in vitro*. Interestingly, PRP-SI could stimulate proliferation of HepG2 cells, maybe the lower degree of sulfation was beneficial to the growth of HepG2 cells.

3.3. Inhibition effect of PRP-S on angiogenesis in zebrafish

Solid tumors require an adequate supply of blood to survive, grow, and metastasize (Carmeliet & Jain, 2000; Hanahan & Folkman, 1996; Li, Shan, Cao, & Dewhirst, 2000). New blood vessels nourishing tumor growth form by angiogenesis, which make inhibition of vessel formation an excellent target for cancer therapy. The zebrafish has become a well accepted model for screening molecules that affect blood vessel formation. Many zebrafish blood vessels form by angiogenic sprouting and appear to require the same proteins that are necessary for blood vessel growth in mammals. It provides the relevance of an *in vivo* environment as well as the potential for high throughput drug screening (Cross, Cook, Lin, Chen, & Rubinstein, 2003; Serbedzija, Flynn, & Willett, 1999). So, the inhibition effects of PRP and its sulfated derivatives on angiogenesis were evaluated in zebrafish. The results were shown in Table 3. It was found that PRP had an obvious inhibition effect on the intersegmental vessel formation of zebrafish with inhibition ratio of 41.07% at 1000 μg/ml, dose-dependently. All sulfated derivatives of PRP could also block new angiogenic and vasculogenic vessels formation, but the effects were weaker than the non-sulfated PRP. At 1000 μg/ml, the inhibition ratios of PRP-SI, PRP-SII, PRP-SIII and PRP-SIV were 26.02%, 21.94%, 27.32% and 14.29%, respectively. The relationship between sulfate content and activity was not observed in the study.

4. Conclusion

Four sulfated derivatives of PRP from *P. ribis* were prepared by chlorosulfonic acid method. Two characteristic absorption bands (at near 1258 and 810 cm^{-1}) appeared in FT-IR spectra, which indicated that the sulfation reaction had actually occurred. The sulfated derivatives had different DS calculated by element analysis. The sulfate groups were not regularly distributed in the polysaccharide chain. PRP-SI with DS of 0.62 could stimulate the growth of HepG2 cells, other sulfated derivatives exhibited obvious inhibition effects on HepG2 cells *in vitro*. All the sulfated derivatives could block new angiogenic vessel formation in zebrafish assay, however, the effects were weaker than the non-sulfated PRP.

Acknowledgements

The authors are deeply grateful to Prof. Kechun Liu and Dr. Sifeng Wang for their help in activities determination, and Dr. Bin Ma and Mrs. Jian Ren for recording the NMR spectra of all samples.

References

Athukorala, Y., Jung, W. K., Vasanthan, T., & Jeon, Y. J. (2006). An anticoagulative polysaccharide from an enzymatic hydrolysate of *Ecklonia cava*. *Carbohydrate Polymers, 66*, 184–191.
Carlucci, M. J., Pujol, C. A., Ciancia, M., Noseda, M. D., Matulewicz, M. C., Damonte, E. B., et al. (1997). Antiherpetic and anticoagulant properties of carrageenans from the red seaweed *Gigartina skottsbergii* and their cyclized derivatives: Correlation between structure and biological activity. *International Journal of Biological Macromolecules, 20*, 97–105.
Carmeliet, P., & Jain, R. K. (2000). Angiogenesis in cancer and other diseases. *Nature, 407*, 249–257.
Chaidedgumjorn, A., Toyoda, H., Woo, E. R., Lee, K. B., Kim, Y. S., Toida, T., et al. (2002). Effect of (1 → 3)- and (1 → 4)-linkages of fully sulfated polysaccharides on their anticoagulant activity. *Carbohydrate Research, 337*, 925–933.
Cross, L. M., Cook, M. A., Lin, S., Chen, N., & Rubinstein, A. L. (2003). Rapid analysis of angiogenesis drugs in a live fluorescent zebrafish assay. *Arteriosclerosis, Thrombosis, and Vascular Biology, 23*, 911–912.
Du, Y. G., Gu, G. F., Hua, Y. X., Wei, G. H., Ye, X. S., & Yu, G. L. (2004). Synthesis and antitumor activities of glucan derivatives. *Tetrahedron, 60*, 6345–6351.
Gamazade, A., Sklyar, S., Nasibov, I., Sushkov, A., Shashkov, Y., & Knirel, Y. (1997). Structural features of sulfated chitosans. *Carbohydrate Polymers, 34*, 113–116.
Han, F., Yao, W., Yang, X., Liu, X., & Gao, X. (2005). Experimental study on anticoagulant and antiplatelet aggregation activity of a chemically sulfated marine polysaccharide YCP. *International Journal of Biological Macromolecules, 36*, 201–207.
Hanahan, D., & Folkman, J. (1996). Patterns and emerging mechanisms of the angiogenic switch during tumorigenesis. *Cell, 86*, 353–364.
Haslin, C., Lahaye, M., Pellegrini, M., & Chermann, J. C. (2001). *In vitro* anti-HIV activity of sulfated cell-wall polysaccharides from gametic, carposporic and tetrasporic stages of the Mediterranean red alga *Asparagopsis armata*. *Planta Medica, 67*, 301–305.
Kimmel, C. B., Ballard, W. W., Kimmel, S. R., Ullmann, B., & Schilling, T. F. (1995). Stages of embryonic development of the zebrafish. *Development Dynamics, 203*, 253–310.
Kovensky, J., Covián, J. A., & Cirelli, A. F. (1990). A suitable method for a rapid estimation of sulphated positions in polysaccharides of pharmacological interest. *Carbohydrate Polymers, 12*, 307–314.
Li, C. Y., Shan, S., Cao, Y., & Dewhirst, M. W. (2000). Role of incipient angiogenesis in cancer metastasis. *Cancer Metals Review, 19*, 7–11.
Lin, Y. L., Zhang, L. N., Chen, L., & Jin, Y. (2004). Molecular mass and antitumor activities of sulfated derivatives of b-glucan from *Poria cocos* mycelia. *International Journal of Biological Macromolecules, 34*, 231–236.
Liu, Y., & Wang, F. (2007). Structural characterization of an active polysaccharide from *Phellinus ribis*. *Carbohydrate Polymers, 70*, 386–392.

Lu, Y., Wang, D., Hu, Y., Huang, X., & Wang, J. (2008). Sulfated modification of epimedium polysaccharide and effects of the modifiers on cellular infectivity of IBDV. *Carbohydrate Polymers, 71*, 180–186.

Mähner, C., Lechner, M. D., & Nordmeier, E. (2001). Synthesis and characterisation of dextran and pullulan sulphate. *Carbohydrate Research, 331*, 203–208.

Martinichen-Herrero, J. C., Carbonero, E. R., Sassaki, G. L., Gorin, P. A. J., & Iacomini, M. (2005). Anticoagulant and antithrombotic activities of a chemically sulfated galactoglucomannan obtained from the lichen *Cladonia ibitipocae*. *International Journal of Biological Macromolecules, 35*, 97–102.

Mossmann, T. (1983). Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. *Journal of Immunological Methods, 65*, 55–63.

Parish, C. R., Freeman, C., Brown, K. J., Francis, D. J., & Cowden, W. B. (1999). Identification of sulfated oligosaccharide-based inhibitors of tumor growth and metastasis using novel in vitro assays for angiogenesis and heparanase activity. *Cancer Research, 59*, 3433–3441.

Preeprame, S., Hayashi, K., Lee, J. B., Sankawa, U., & Hayashi, T. (2001). A novel antivirally active fucan sulfate derived from an edible brown alga, *Sargassum horneri. Chemical Pharmaceutical Bulletin, 49*, 484–485.

Qiu, H., Tang, W., Tong, X., Ding, K., & Zuo, J. (2007). Structure elucidation and sulfated derivatives preparation of two α-D-glucans from *Gastrodia elata* Bl. and their anti-dengue virus bioactivities. *Carbohydrate Research, 342*, 2230–2236.

Richter, A., & Wagenknecht, W. (2003). Synthesis of amylose acetates and amylose sulfates with high structural uniformity. *Carbohydrate Research, 338*, 1397–1401.

Serbedzija, G. N., Flynn, E., & Willett, C. (1999). Zebrafish angiogenesis: A new model for drug screening. *Angiogenesis, 3*, 353–359.

Shi, B. J., Nie, X. H., Chen, L. Z., Liu, Y. L., & Tao, W. Y. (2007). Anticancer activities of a chemically sulfated polysaccharide obtained from *Grifola frondosa* and its combination with 5-fluorouracil against human gastric carcinoma cells. *Carbohydrate Polymers, 68*, 687–692.

Vogl, H., Paper, D. H., & Franz, G. (2000). Preparation of a sulfated linear (1 → 4)-β-D-galactan with variable degrees of sulfation. *Carbohydrate Polymers, 41*, 185–190.

Williams, D. L., Pretus, H. A., McNamee, R. B., Jones, E. L., Ensley, H. E., Browder, I. W., et al. (1991). Development, physicochemical characterization and preclinical efficacy evaluation of a water soluble glucan sulfate derived from *Saccharomyces cerevisiae*. *Immunopharmacology, 22*, 139–156.

Wu, X. Z., Chen, D., & Xie, G. R. (2006). Effects of Gekko sulfated polysaccharide on the proliferation and differentiation of hepatic cancer cell line. *Cell Biology International, 30*, 659–664.

Yang, J., Du, Y., Yan, W., Li, T., & Hu, L. (2003). Sulfation of Chinese lacquer polysaccharides in different solvents. *Carbohydrate Polymers, 52*, 397–403.

Yoshida, T., Yasuda, Y., Mimura, T., & Kaneko, Y. (1995). Synthesis of curdlan sulfates having inhibitory effects in vitro against AIDS viruses HIV-1 and HIV-2. *Carbohydrate Research, 276*, 425–436.

Zhou, G., Sun, Y., Xin, H., Zhang, Y., Li, Z., & Xu, Z. (2004). In vivo antitumor and immunomodulation activities of different molecular weight lambda-carrageenans from *Chondrus ocellatus*. *Pharmacological Research, 50*, 47–53.