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Sulfation of (1→3)-β-D-glucan from the fruiting bodies of *Russula virescens* and antitumor activities of the modifiers

Zhongwei Sun, Yunlong He, Zhihong Liang, Wenwen Zhou, Tiangui Niu *

College of Food Science and Nutritional Engineering, China Agricultural University, P.O. Box 111, No. 17 Qinghua East Road, Beijing 100083, China

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ABSTRACT

A water-insoluble (1→3)-β-D-glucan isolated from the fresh fruiting bodies of *Russula virescens* was sulfated using sulfur trioxide-pyridine complex as reagent in dimethyl sulfoxide. Depending on the reaction conditions, the products showed different degrees of sulfation (DS) ranging from 0.17 to 1.17 and different weight average molecular weights (M_ws) ranging from 2.5×10^4 to 1.2×10^5 Da. Moreover, the antitumor activities of the five sulfated derivatives against Sarcoma 180 tumor cell were tested both *in vitro* and *in vivo*. The results indicated that the native (1→3)-β-D-glucan did not show antitumor activity, while the sulfated derivatives exhibited enhanced antitumor activities. This study demonstrated that DS and M_w could influence the antitumor activities of the sulfated derivatives.

1. Introduction

In recent years, polysaccharides and their sulfated derivatives have attracted much attention due to their diverse biological activities. Sulfated polysaccharide, either natural or chemically synthesized, is a kind of one with sulfated group in its hydroxyls. Many studies confirmed that sulfated polysaccharides have stronger biological activities. Consequently, sulfation is utilized to improve the biological activities of some polysaccharides as well as obtain more sulfated polysaccharides. Sulfation of polysaccharides has been reported for a variety of polysaccharides, such as fucoidan (Teruya, Konishi, Uechi, Tamaki, & Tako, 2007), curdlan (Alban & Franz, 2000; Fritzsche et al., 2006; Koumoto et al., 2004), fucan (Ferial, Mostafa, Corinne, & Catherine, 2000), chitosan (Gamzazade et al., 1997; Vikhorevaa et al., 2005; Xing et al., 2004), and pullulan (Alban, Schauerte, & Franz, 2002; Fritzsche et al., 2006; Mihai, Mocanu, & Carpov, 2001). In fact, many methods have been demonstrated to sulfate polysaccharides, examples of which are via the use of sulfuric acid, chlorosulfonic acid–pyridine, sulfur trioxide–pyridine (SO_3·Py), and sulfur trioxide–dimethylacetamide, and so on.

Russulus virescens is a wild mushroom that grows on the roots of pine trees. In present paper, we isolated a water-insoluble linear (1→3)-β-D-glucan, named as RVS3-II, from the fresh fruiting bodies of *Russula virescens*. Evidence from different studies suggested that the biological activities of sulfated polysaccharides would strongly depend on their structure, the degree of sulfation (DS), the molecular weight, the sulfation pattern, and the glycosidic branches. In the present study, RVS3-II was modified by sulfation with SO_3·Py complex to prepare a variety of sulfated polysaccharides. Only a few papers have investigated the influence of reaction temperature, reaction time, and the amount of reaction reagent on the structure of sulfated derivatives. In the current work, these factors affecting sulfation of RVS3-II were studied. Moreover, the polymer characteristics of the sulfated derivatives and the native polysaccharide, including weight average molecular weight (M_w) and DS, as well as *in vitro* and *in vivo* antitumor activities were investigated.

2. Experimental

2.1. Materials

The sulfation reagent used was SO_3·Py, which was purchased from Tokyo Kasei Kegyo Co., Ltd (Tokyo, Japan). T-series Dextrans were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Dimethyl sulfoxide (DMSO) was first treated with 5 Å molecular sieve to remove water, and was afterwards distilled under reduced pressure. All other chemicals employed were of analytical grade with no further purification done.

2.2. Isolation and purification of RVS3-II

The fresh fruiting bodies of *R. virescens* were dried and ground. The dried powder was defatted by using Soxhlet extraction with ethyl for 8 h and then acetone for 6 h to remove lipophilic com-

* Corresponding author. Tel.: +86 10 62737045.
E-mail address: globlefood@163.com (T. Niu).

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pounds, and then successively boiled in distilled water at 100 °C for 4 h and then contrifugated. The residue from the centrifugation was extracted three times with hot water at 100 °C for 4 h. After centrifugation to remove the supernatant, the residue was treated with 0.5 M NaOH for 24 h at 4 °C. The resultant syrup was centrifuged at 10,000 rpm for 20 min to obtain the supernatant, which was later decolorized with 30% H_2O_2, deproteinated 10 times by the Sevag method (Sevag, 1934), and then dialyzed using regenerated cellulose tube (molecular weight cut-off 8000 Da, Union carbide, NJ, USA) against tap water for five days and then distilled water for four days. The resultant solution was neutralized with acetic acid and centrifuged to obtain the sediment fraction named as RVS3-II.

2.3. Preparation of sulfated derivatives

Sulfation of RVS3-II was carried out by initially dissolving/suspending the RVS3-II powder in dry solvent, and the mixture was stirred at reaction temperature for 30 min. Subsequently, SO_3·Py complex was added. Various molar ratios of the SO_3·Py complex to sugar residues and their reaction durations were used. After cooling to room temperature, the mixture was neutralized by addition of a saturated $NaHCO_3$ solution, followed by dialysis against deionized water for 120 h with periodic bath changes to remove unreacted compounds. The dialyzate was then concentrated under a reduced pressure below 45 °C and was freeze-dried from water to obtain the sulfated derivatives.

2.4. Characterization

2.4.1. FT-IR spectra

The infrared spectra of the polysaccharides were recorded with a Nicolet NEXUS-470 FT-IR (Spectrum One, Perkin Elmer Co., USA) spectrometer in the range of 4000–500 cm^{-1} using the KBr disk method.

2.4.2. NMR spectra

The ^{13}C nuclear magnetic resonance (NMR) spectra were obtained with the use of a 400 MHz Bruker model DRX Avance spectrometer. All the samples were kept over P_2O_5 in vacuo for two days and then were deuterium-exchanged by freeze-drying three times from D_2O, followed by lyophilization with D_2O. The ^{13}C NMR analyses were performed at 30 °C, with the samples dissolved in D_2O for S-RVS3-II or DMSO-d_6 for RVS3-II, depending on their solubility characteristics.

2.4.3. Determination of molecular weights

The M_ws of RVS3-II and the sulfated derivatives were evaluated and determined by HPLC on two Water Ultrahydrogel TM Linear 7.8 × 300 mm columns and were eluted with 0.1 mol L^{-1} of sodium nitrate solution at a flow rate of 0.9 mL min^{-1} at 45 °C. Elution was monitored by a refractive index detector. The column was calibrated with glucose (Molecular weight: 180 Da) and T-series Dextran standards (molecular weight: 4600, 10,000, 21,400, 41,100, 133,800, 482,000, 2,000,000 Da). Individual molecular weight standard solutions were prepared fresh daily by dissolving 25 mg each of the eight standards from 180 to 2,000,000 Da in 50 mL DMSO. RVS3-II and the sulfated glucan derivative solutions were prepared in a similar fashion. The standard solutions were then injected in duplicate in the order of decreasing molecular weight, with the GPC software (Thermo Labsystems, Shrewsbury, MA, USA) used to perform a narrow band linear regression standard calibration curve of log molecular weight versus HPLC retention time. The sample preparations were afterwards injected in duplicate.

2.5. Antitumor test

2.5.1. In vivo antitumor test

Sarcoma 180 tumor cells were subcutaneously inoculated (2×10^6 cells/mouse) into 8-week-old female BALB/c mice. Subsequently, 5-Fluorouracil (5-Fu) and the tested samples were dissolved in phosphate buffer saline (PBS; 8.812 g NaCl, 0.201 g KCl, 0.204 g KH_2PO_4, and 1.150 g Na_2HPO_4 were dissolved in 1 L of distilled water), and injected intraperitoneally (ip) once a day for 10 days, starting at 24 h after tumor inoculation. The same volume of PBS was injected ip into the control mice. The mice were sacrificed on the next day after the last injection, and the tumors were excised. The tumor weights were compared with those in the control mice. The inhibition ratio (ξ) and enhancement ratio of body weight (f) were calculated as follows:

$$\xi = [(W_c - W_t)/W_c] \times 100\% \quad (1)$$

$$f = [(W_a - W_b)/W_b] \times 100\% \quad (2)$$

where W_c is the average tumor weight of the control group, W_t is the average tumor weight of the test group, and W_b and W_a are the body weight of mice before and after the assay. The statistical evaluations in all experiments were performed by the Student t-test. A P value of less than 0.05 was considered significant.

2.5.2. In vitro antitumor test

The colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method was used to measure the proliferation of adherent tumor cells. The Sarcoma 180 tumor cells were inoculated on a 96-well cultivation plate at a concentration of 1×10^4 cells/mL. Each well was inoculated with 100 μL Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 10% fetal bovine serum solution containing the tumor cells and 20 μL samples (at concentrations of 0.005, 0.05, 0.5, and 5 mg/mL in PBS, respectively) under an atmosphere of 5% CO_2 at 37 °C for 24 h. The tumor cells were continuously inoculated for another 4 h after 10 μL MTT (5 mg/mL) had been added. The supernatant was removed by centrifugation at 1000 rpm for 10 min at 4 °C, and 100 μL DMSO was added to terminate the reaction. The survival rate of the tumor cells was assayed through measurement of optical intensity by an auto enzyme-labeled meter at 550 nm. The sample groups were compared with the control group in the absence of the test samples. All *in vitro* results were expressed as the inhibition ratio (Φ) of tumor cell proliferation as follows:

$$\Phi = [(A - B)/A] \times 100\% \quad (3)$$

where A and B are the average number of viable tumor cells of the control group and test group, respectively. All assays were made in triplicate (Wang, Zhang, Li, Hou, & Zeng, 2004).

3. Results and discussion

3.1. Chemical structure

The FT-IR spectra of the native RVS3-II and the sulfated derivatives are illustrated in Fig. 1. Compared with the FT-IR spectrum of native RVS3-II, two new absorption peaks appeared at around 1261 and 820 cm^{-1} for the sulfated derivatives, which are due respectively to the presence of the S═O and C—O—S groups associated to the C—O—SO_3 group. This then indicated that sulfated reaction had actually occurred (Yang, Du, Huang, Wan, & Wen, 2005).

The ^{13}C NMR spectra of native RVS3-II and sulfated derivative are demonstrated in Fig. 2. The ^{13}C spectrum of RVS3-II exhibited only six strong chemical shifts at δ = 102.7, 86.1, 75.6, 73.1, 68.2, and 60.8, assigned as C-1, C-3, C-5, C-2, C-4, and C-6. The results

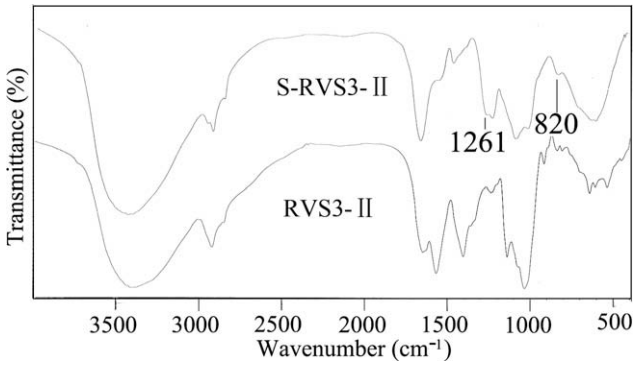

Fig. 1. FI-IR spectra of RVS3-II and its sulfated polysaccharides.

indicated RVS3-II was a linear (1→3)-β-D-glucan with high purity (Carboneroa et al., 2006; Elaine et al., 2006; Krishnendu et al., 2007). Consequently, the strong signals around 103.1, 85.3, 76.6, 73.6, 69.5, and 61.4 ppm for S-RVS3-II were assigned as C-1, C-3, C-5, C-2, C-4, and C-6 of (1→3)-β-D-glucan. Compared with the ^{13}C NMR spectrum of the native RVS3-II sample, there were five new peaks that appeared in the S-RVS3-II spectrum, namely, C-1′ (101.8), C-2s (79.8), C-4s (75.7), C-5′ (74.3), and C-6s (67.7 ppm). These resulted from sulfation of the hydroxyl groups at positions 6, 4, and 2. A new peak also appeared at 67.7 ppm in the S-RVS3-II spectrum, assigned to the substituted C-6, because the downfield shift of a carbon atom linked by a sulfated group is ~7–10 ppm. The peak of C-5 then shifted from $\delta = 76.7$ to 74.3 (C-5′). Similarly, the new peaks at 79.8 and 75.7 ppm were respectively assigned to the substituted C-2 and C-4. Moreover, a new peak at 101.8 ppm could be assigned to C-1′ because C-2 and C-6 were substituted to influence the chemical shift of the adjacent C-1, leading to the splitting of the C-1 carbon signal. From the results of ^{13}C NMR, it is reasonable to conclude that the sulfation of RVS3-II has occurred with the substitution of C-2, C-4, and C-6.

3.2. Effects of various reaction conditions on degrees of sulfation

The sulfate group plays an important role in the bioactivities of polysaccharides. The DS of polysaccharides is also an important bioactivity parameter. With this in mind, it is therefore interesting to test the effects of molar ratio of SO_3·Py complex to sugar unit, reaction time, and reaction temperature on the DS of the sulfated derivatives, respectively. The DS that designates the average number of sulfur groups on each sugar residue was calculated from the sulfur content (S%) by the following formula (Schoniger, 1956):

$$DS = (S\% \times 162)/(32 - 102 \times S\%) \quad (4)$$

In order to generate sulfated polysaccharides with different DS, the different molar ratios of the SO_3·Py complex to the sugar unit (1:1, 2:1, 3:1, 4:1) were used. For every molar ratio, the reaction was conducted at 60 °C for 3 h. Fig. 3 illustrates that as the molar

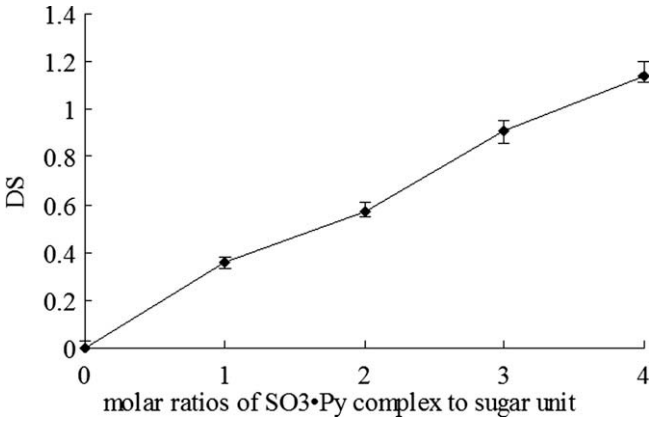

Fig. 3. Influence of the molar ratio of SO_3·Py complex to sugar unit on DS at 60 °C for 3 h. Each data point is a mean ± standard deviation of three replicated determinations.

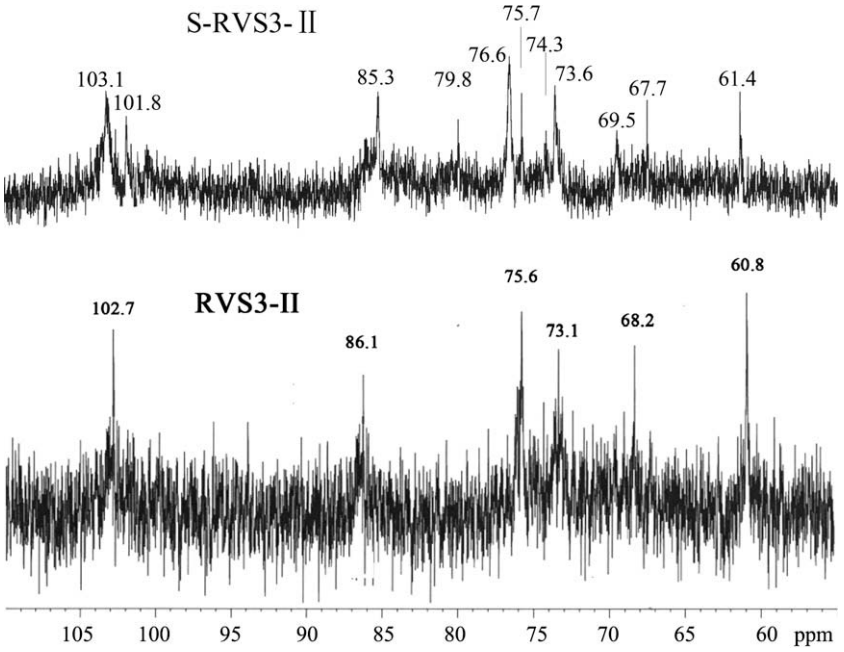

Fig. 2. ^{13}C NMR spectra of RVS3-II and S-RVS3-II.

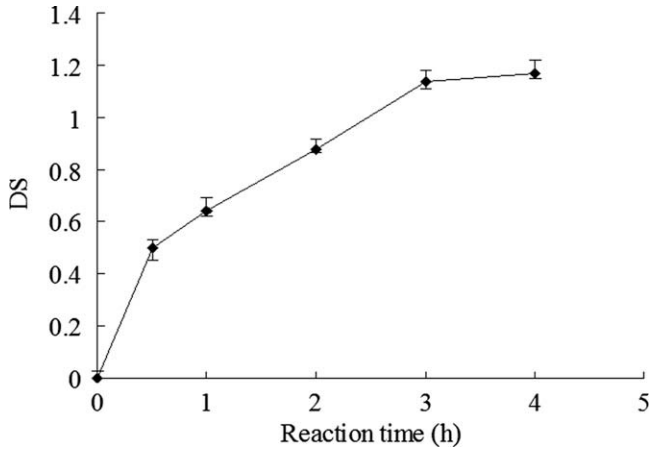

Fig. 4. Effects of reaction time on DS with 4:1 molar ratio of SO_3·Py complex to sugar unit at 60 °C. Each data point is a mean ± standard deviation of three replicated determinations.

ratio of the SO_3·Py complex to the sugar unit increased, the DS of the modified polysaccharides also increased. The DS was observed to range from 0.35 to 1.14.

The effects of reaction time were also investigated. Six products were prepared by varying reaction time with the 4:1 molar ratio of the SO_3·Py complex to the sugar unit at 60 °C. The results in Fig. 4 show that the maximal DS (the values of which depend on the temperature used) was attained after 4 h.

The influence of temperature (ranging from 40 to 100 °C) on DS was also investigated (Fig. 5). For every reaction temperature, the reaction was conducted with a 4:1 molar ratio of the SO3·Py complex to the sugar unit for 3 h. The DS increased with reaction temperature up to 60 °C. However, higher reaction temperature reduced the DS value.

3.3. Change in M_ws during sulfation

The M_w of the sulfated polysaccharide is another important parameter influencing polysaccharide bioactivities. The sulfation reaction process of polysaccharides is usually accompanied by degradation. Thus, maintenance of the macromolecular features of the original polysaccharides during sulfation reaction is an important research field. In this study, The M_ws of the sulfated polysaccharide were evaluated by high performance liquid chromatography (HPLC). Fig. 6 illustrates a plot of log (Mol Wt) of the standard samples versus HPLC retention time. A regression line with good correlation coefficient was then obtained (Log Mol Wt = 13.6 − 0.521T, where T was the retention time, R^2 = 0.996).

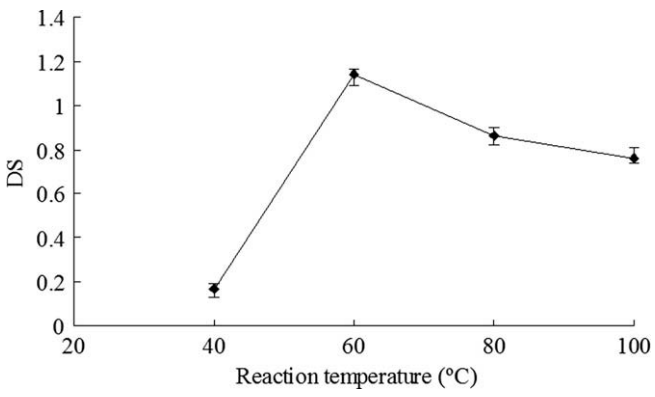

Fig. 5. Effects of reaction temperature on DS with 4:1 molar ratio of SO_3·Py complex to sugar unit for 3 h. Each data point is a mean ± standard deviation of three replicated determinations.

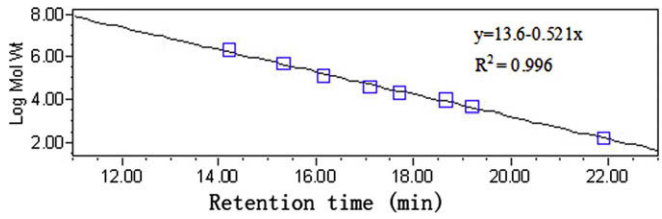

Fig. 6. A plot of log (Mol Wt) of the standard samples versus HPLC retention time.

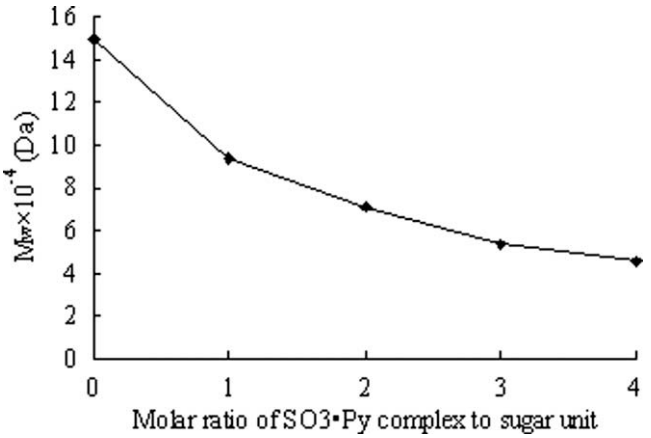

Fig. 7. Influence of the molar ratio of SO_3·Py complex to sugar unit on M_w at 60 °C for 3 h.

Fig. 7 presents the effects of the molar ratio of the SO_3·Py complex to the sugar unit on M_w at 60 °C for 3 h. The mass of the sulfation reagent greatly influenced the M_ws of products, that is, the higher the reagent to the sugar unit ratio was used, the lower the sulfated derivatives' M_ws were. For instance, when the molar ratio was 4:1, the M_w dropped form 1.5×10^5 to 4.6×10^4 Da.

Fig. 8 plots the effects of reaction time on M_w when the 4:1 molar ratio of the SO_3·Py complex to the sugar unit was used in all cases at 60 °C. In the first hour, the M_w decreased significantly. This could be due to the concentration of the SO_3·Py complex being higher in the primary stage, or the easy degradation of polysaccharides with low DS or absent sulfation.

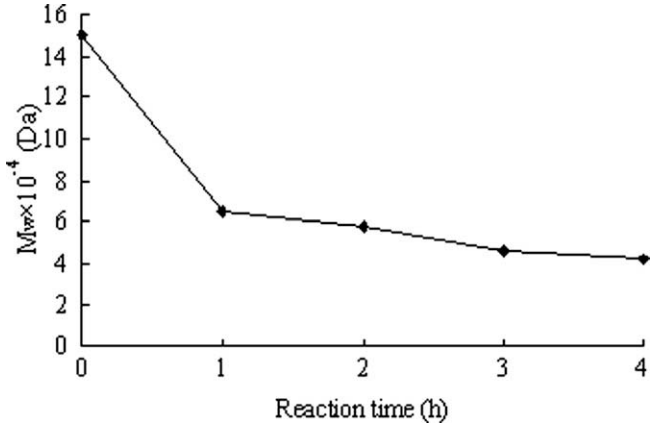

Fig. 8. Effects of reaction time on M_w with 4:1 molar ratio of SO_3·Py complex to sugar unit at 60 °C.

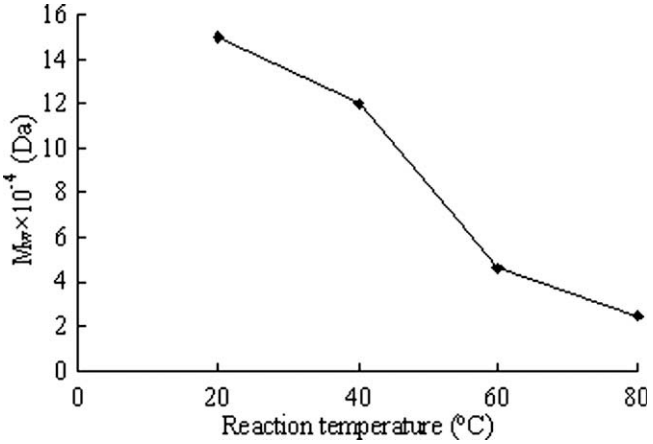

Fig. 9. Influence of reaction temperature on M_w with 4:1 molar ratio of SO_3·Py complex to sugar unit for 3 h.

The influence of temperature (ranging from 20 to 80 °C) on M_w was also investigated (Fig. 9). For every reaction temperature, the reaction was conducted with a 4:1 molar ratio of the SO_3·Py complex to the sugar unit for 3 h. When the temperature was higher than 40 °C, the M_ws of the sulfated derivatives decreased promptly.

Table 1
Antitumor activities *in vivo* of RVS3-II and five sulfated derivatives against Sarcoma 180 solid tumor cells grown in BALB/c mice.

Samples	Dose (mg/kg × days)	Inhibition ratio (%)	Enhancement ratio of body weight (%)	M_w (Da)	DS
RVS3-II	16 × 10	2.84	42.24	1.5×10^5	0
	32 × 10	3.56	48.69		
S-RVS3-II-1	16 × 10	33.45[a]	42.86	4.6×10^4	1.14
	32 × 10	37.26[a]	49.58		
S-RVS3-II-2	16 × 10	35.73[a]	43.45	5.8×10^4	0.88
	32 × 10	31.42[a]	40.22		
S-RVS3-II-3	16 × 10	26.74	43.31	4.2×10^4	1.02
	32 × 10	30.54	46.53		
S-RVS3-II-4	16 × 10	14.27	43.42	1.2×10^5	0.17
	32 × 10	9.31	50.31		
S-RVS3-II-5	16 × 10	17.12	44.28	2.5×10^4	0.86
	32 × 10	23.97	37.65		
Control		0	41.78		
5-Fu	16 × 10	51.6[b]	13.2		

[a] Compared with the control group $P < .05$.
[b] Compared with the control group $P < .001$.

3.4. Antitumor activities

Considering the M_ws and DS, five sulfated derivatives and the native RVS3-II were chosen to evaluate their *in vivo* antitumor activities against Sarcoma 180 tumor cell. The results of the *in vivo* assay of RVS3-II and the five sulfated derivatives in different doses against solid Sarcoma 180 tumor cells implanted in BALB/c mice are summarized in Table 1. A well-known anticancer agent, 5-Fu, was included for comparison. The native RVS3-II was then determined to exhibit no antitumor activity against Sarcoma 180 tumor cell, although all the sulfated derivatives tested showed higher antitumor activities than the native one. This suggested that antitumor activities could be improved by sulfation. Moreover, the enhancement ratios of the body weight of mice injected with the sulfated derivatives were higher than those injected with 5-Fu, implying that the sulfated derivatives did not have the same toxicity as 5-Fu, which kills normal cells as well as cancer cells. Macrofungal polysaccharides are regarded as biological response modifiers that cause no harm and place no additional stress on the body. Instead, they help the body adapt to various environmental and biological stresses. In addition, macrofungal polysaccharides do not attack tumor cells directly but produce antitumor effects by activating different immune responses in the host (Wasser, 2002).

The inhibition ratios to the proliferation of Sarcoma 180 tumor cells by different concentrations (0.005, 0.05, 0.5, and 5 mg/mL) of RVS3-II and the five sulfated derivatives are shown in Fig. 10. In contrast, the five sulfated derivatives all exhibited relatively enhanced inhibitions against Sarcoma 180 tumor cell growth at all concentrations. The S-RVS3-II-1 exhibited the highest inhibition ratios of 11, 35, 41, and 43% to the proliferation of Sarcoma 180 tumor cell line at the concentrations of 0.005, 0.05, 0.5, and 5 mg/mL, respectively. However, no obvious dose-dependence relationship was observed between the concentration of derivatives and the growth inhibition of Sarcoma 180 tumor cell. The derivatives' *in vitro* antitumor activities against Sarcoma 180 tumor cell are derived from the stimulation of the immune response mechanism. Therefore, they do not strictly follow the dose-dependent characteristic of chemotherapeutic anticancer agents.

4. Conclusions

Sulfated derivatives were satisfactorily synthesized from water-insoluble (1→3)-β-D-glucan extracted from the fruiting bodies of *Russula virescens* by way of the SO_3·Py complex as reagent in DMSO. Depending on the reaction conditions, the products showed

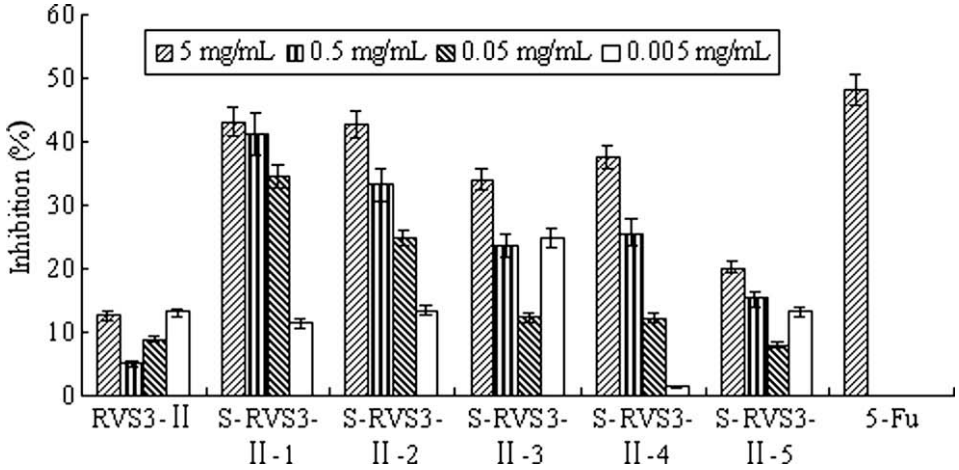

Fig. 10. Inhibition ratio of the proliferation of Sarcoma 180 tumor cells by different concentrations of RVS3-II and five sulfated derivatives.

different DS ranging from 0.17 to 1.17, and different M_ws ranging from 2.5×10^4 to 1.2×10^5 Da. The antitumor activities of the five sulfated derivatives against Sarcoma 180 tumor cell were likewise tested both *in vitro* and *in vivo*. It was determined that the sulfation of the native (1→3)-β-D-glucan could improve its antitumor activity.

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