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Sulfated modification of the polysaccharide from *Cordyceps_gunnii* mycelia and its biological activities

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ABSTRACT

A chemically new sulfated polysaccharide (SPS50) was prepared from the water soluble polysaccharide (PS50), isolated from *Cordyceps_gunnii* mycelia, by concentrated sulfuric acid method. The yield of crude SPS50 was 62.34% and its specific rotation was $[\alpha]_D^{20} = -36.75^\circ$. The structural characteristics of this chemically sulfated polysaccharide were determined based on the infrared analysis (IR), high performance liquid chromatography (HPLC) and sodium dodecyl sulfate polyacrylamide gel electropheresis (SDS-PAGE). Its biological properties including anti-oxidant and anti-tumor activities were also investigated. The results showed that the anti-oxidant capacity of SPS50 was not as good as SP50 and the anti-tumor activity of SPS50 was much better than PS50. SPS50 showed evident growth inhibition on K562 cells. The tumor inhibition ratio of SPS50 against K562 cells was 69.92%.

1. Introduction

Polysaccharide has varieties of functions and biological activities, such as anti-cancer, anti-oxidant (Chen et al., 2005; Martinichen-Herrero, Carbonero, Sassaki, Gorin, & Iacomini, 2005). In recent years, an increasing number of studies have focused on the structures and biological properties of sulfated polysaccharides (Han, Yao, Yang, Liu, & Gao, 2005; Yang, Du, Huang, Sun, & Liu, 2005; Urbinati, Bugatti, & Oreste, 2004; Xing et al., 2005; Lee, Bae, & Pyo, 2003). It was demonstrated that chemically sulfated polysaccharides might change the chain conformation, resulting in the alteration of their biological activities (Wang, Zhang, Li, Hou, & Zeng, 2004; Wang, Yu, & Yuan, 2004).

Cordyceps gunnii (Berk). Berk., with two life forms of sexual and asexual types, is one kind of a complex type of fungi, and is found in Australia and China's Guizhou, Anhui, Guangdong, Jiangxi and Hunan provinces. Earlier results indicated that *C. gunnii* could improve the body immunity, increasing memory, delay senescence, protect brain and heart in the condition of atmospheric oxygen, etc. (Wang, Zhang, et al., 2004; Wang, Yu, et al., 2004; Liang, 1985).

Polysaccharides from *C. gunnii* in biological systems were reported to possess several bioactivities, such as anti-oxidation, anti-tumor, anti-aging activities and capable of increasing immunity potential (Xu, 2002; Zhu et al., 2011). In our previous study, a water soluble polysaccharide named PS50 (Zhu et al., 2012) was extracted from *C. gunnii*. Thus the obtained polysaccharide had several bioactivities, and can be used as a dietary supplement for health foods and therapeutics.

In this paper, a new chemically sulfated polysaccharide was prepared from the water soluble polysaccharide (PS50) (Zhu et al., 2012), named SPS50. The SPS50's structural characteristics were determined by IR, HPLC and SDS-PAGE. The anti-oxidation and anti-tumor activity of SPS50 were also investigated.

2. Materials and methods

2.1. Materials

PS50, a high-molecular-weight polysaccharide, was isolated from *C._gunnii* mycelia based on our previous work (Zhu et al., 2012). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and sodium dodecyl sulfate (SDS) were purchased from Sigma (St. Louis, MO, USA). All other chemicals used in this paper were of analytical grade.

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2.2. Preparation and isolation of SPS50

The sulfated polysaccharide was prepared using concentrated sulfuric acid (7.5 mL), n-butanol (12.5 mL) as described previously (He, Chen, & Li, 2003). Ammonium sulphate (125 mg) was added into 50 mL three-necked bottle. And then 500 mg PS50 sample was suspended and added. The mixture was maintained at 0 °C for 30 min with continuous stirring. After the reaction was finished, the mixture was neutralized with 1.0 mol/L NaOH dialyzed against distilled water for 3 d, and freeze dried. A fraction of sulfated polysaccharides was obtained and named as SPS50.

The fraction of sulfated polysaccharide was loaded on to a column (1.6 cm × 30 cm) of DEAD sephadex A-25. It was followed by eluting at a flow rate of 0.5 mL/min successively with distilled water and a gradient of 0–1.5 mol/L NaCl solution (each tube 2 mL). The carbohydrate content of the elute was determined by the phenol–sulfuric acid method. Fraction collected from the main peak subjected to freeze drying.

2.3. The degree of sulfate substitution (DS)

The sulfate content (S%) in the sulfated derivative was tested according to the method of barium sulfate turbidity (Antonopoulos, 1962). The degree of DS, designated as the average number of O-sulfate groups per residue was calculated from the sulfur content by the following formula (Zhang, 1999):

$$DS = \frac{1.62 \times S\%}{32 - 1.02 \times S\%}$$

2.4. IR analysis

One milligram of dry SPS50 was mixed with 150 mg of dry KBr and pressed into a disk for spectrum recording. The Fourier transform IR spectra (FT-IR) were recorded on a Vector 22 FT-IR spectrophotometer (BioRad FT S-135) between 400 and 4000 cm^{-1}.

2.5. Analysis by HPLC

The SPS50 was analyzed with a HPLC (Agilent-1100) equipped with a C_{18} column (300 mm × 20 mm) and a 200 nm UV detector, at detecting temperature 30 °C. 20 μL of sample solution was injected and ran with phosphate buffer (pH 6.0) at 0.6 mL/min as mobile phase.

2.6. SDS-PAGE fibrinautography

The purity of sulfated polysaccharides was further confirmed by SDS polyacrylamide slab gel electrophoresis. It was configured by 18% (v/v) resolving gel, 10% (v/v) stacking gel, the sample solution pretest treatment. A constant pressure of 80 V/cm was used. Colored by alixinlan solution for 4 h, glacial acetic acid:methanol = 1:4 as the decolorize for 24 h. (I do not understand this! First you colored it and then discolored it?)

2.7. Anti-oxidant activity of SPS50

2.7.1. Superoxide radical (SR) scavenging assay

Superoxide radical was generated in the PAPG system containing 340 μL Tris–HCl buffer (50 mM, pH 8.2), 50 μL25 mM, PAPG and 100 μL varying concentrations of polysaccharide (2.5–15 mg/mL). The mixture prepared earlier was incubated at 25 °C for 4 min. Subsequenlty, 10 μL 8 mM HCl was used to compete the reaction. The absorbance reading at 320 nm was named as A_i. For the positive control, sample was substituted with V_c and the absorbance reading at 320 nm was named as A_j. In the blank group, sample was substituted with distilled water and the absorbency value was named as A_o. The capability of scavenging to superoxide radical was calculated using the following equation:

$$SR(\%) = \left[1 - \frac{A_i - A_j}{A_o}\right] \times 100\%$$

2.7.2. 1,1-Diphenyl-2-picrylhydrazyl scavenging assay (DPPH·)

The free-radical scavenging capacity of the sulfated polysaccharides (SPS50) was analyzed using the DPPH·test according to the method of Blois (1958). 100 μL of various concentrations of the sulfated polysaccharides (2.5–15 mg/mL) were added to 400 μL of 5 μmol/L 50% (v/v) ethanol solution of DPPH·. After a 30 min incubation period at 25 °C, the absorbance was determined against a blank at 517 nm. The free radical inhibition by DPPH in percent (SR%) was calculated as follows:

$$SR(\%) = \left[1 - \frac{A_i - A_j}{A_o}\right] \times 100\%$$

2.8. Hydroxyl radical scavenging assay

The scavenging activity of water soluble polysaccharide of *C. gunnii* against the hydroxyl radical was investigated based on the Fenton reaction ($Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + OH$), from which high reactive hydroxyl radicals, ·OH, are generated.

The reaction solution, containing 200 μL different concentrations (2.5–15 mg/mL) was incubated with 6 mM (200 μL) salicylic acid, 6 mM H_2O_2 (200 μL), 6 mM (200 μL) ferrous sulfate for 60 min at 37 °C and hydroxyl radical was detected by monitoring absorbance at 510 nm. In the control, sample was substituted with distilled water and the same concentration of V_c replaced H_2O_2. The capability of scavenging hydroxyl radical was calculated as:

$$SR\% = \left[\frac{A_o - (A_i - A_j)}{A_o}\right] \times 100\%$$

2.9. Assay of cell growth (anticancer activity of SPS50)

K562 cells were obtained from Tianjin University of Science and Technology biological resources and function laboratory (Tianjin, China). The cells were cultured in RPMI1640 medium containing 10% FBS in a humidified atmosphere of 5% CO_2 at 37 °C. The cell growth was determined by the MTT cellular viability assay method (Liu, Song, Yang, Liu, & Zhang, 2007; Liu, Lin, Gao, Ye, & Xi, 2007). 2×10^5 K562 cells were placed into 96-well microplates and cultured for 24 h prior to treatment. The cells were treated with various concentrations of SPS50. At the end of treatment, 10 μLMTT solution (5 mg/mL) was added to each well and incubated for another 4 h. The medium was removed and 150 μl dimethylsulfoxide (DMSO) was added to the wells. Then the absorbance was measured at 490 nm using an ELISA reader (Bio-Tek EL 800, USA). All determinations were conducted in triplicate. The inhibition of cell growth was calculated by following formula:

$$\text{Inhibition rate}(\%) = \frac{A_{control} - A_{sample}}{A_{control}} \times 100\%$$

3. Results and discussion

3.1. Structure analysis

The SPS50 was prepared from PS50 by concentrated sulfuric acid method. The yield of crude SPS50 was 62.34%. The crude SPS50 was

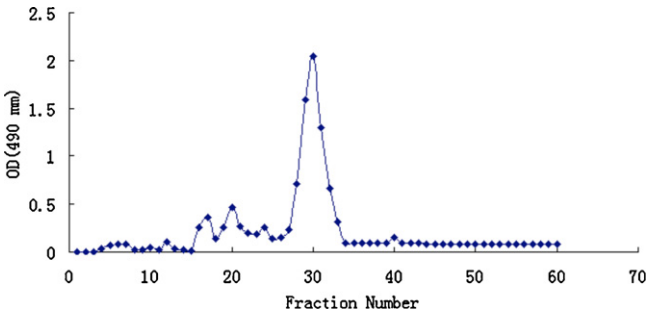

Fig. 1. DEAE Sephadex A-25 chromatographic profile for SPS50.

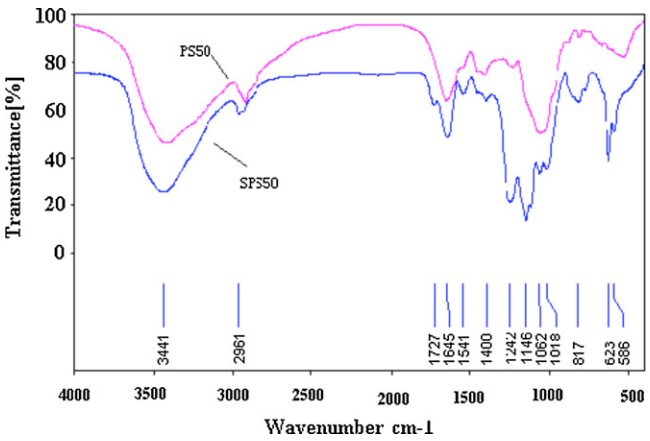

Fig. 2. A comparison of PS50 and SPS50 IR spectra (PS50 at top and SPS50 at bottom).

purified by DEAE Sephadex A-25, saving the main fraction (Fig. 1) which was then freeze dried. The specific rotation of SPS50 was recorded as $[\alpha]_D^{20} = -36.75^\circ$.

The sulfur content in SPS50 was 5.9% (w/w), thus, the degree of substitution (DS) was 0.33.

By comparison with PS50 (Fig. 2), two characteristic absorption bands appeared in the FT-IR spectrum of SPS50 one at 1241 cm^{-1}, which was due to an asymmetrical S=O stretching vibration and the other at 817 cm^{-1} due to a symmetrical C—O—S vibration associated with a C—O—SO_3 group, indicating incorporation of the sulfate group (Maciel, Chaves, & Souza, 2008; Mahner, Lechner, & Nordmeier, 2001).

The high-performance liquid chromatography analysis of PS50 was shown in Fig. 3a. PS50 appeared a primary peak and the retention time was 2.833 min.

Compared with the PS50 HPLC chart, SPS50 exhibited two primary peaks with the retention time of 2.833 and 4.667 min respectively (Fig. 3b).

The results also indicated that a part of PS50 had been sulfated successfully and the polarity of SPS50 was larger than PS50.

Alcian Blue, a cationic dye is the most specific dye on acidic mucous material. Dyes and acid groups can form a salt. When acidic polysaccharides were mixed with alcian Blue a different color appeared. However, when acidic polysaccharides were replaced with neutral polysaccharides, the color of the alcian Blue kept the same.

In Fig. 4, the neutral polysaccharide PS50 did not show significant band in SDS-PAGE. But the sulfated SPS50 had a wide band.

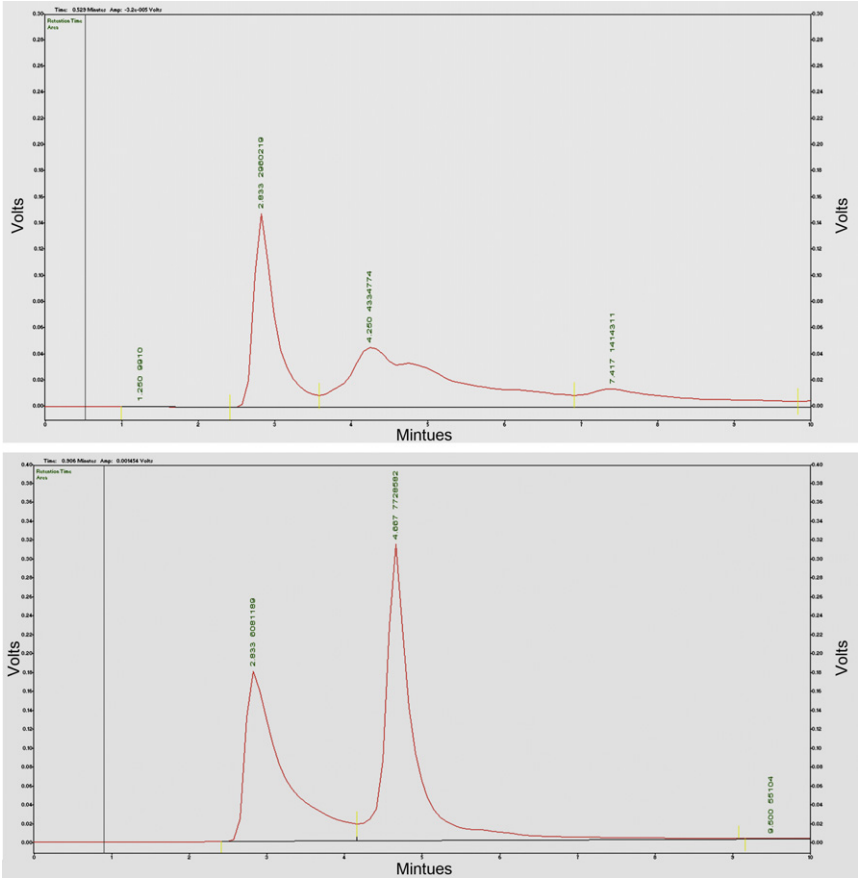

Fig. 3. HPLC profile of PS50 (a) and SPS50 (b).

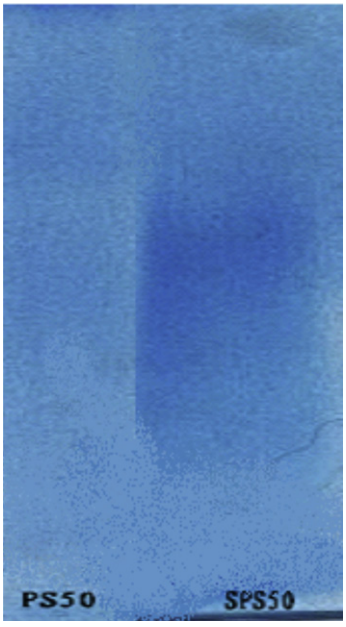

Fig. 4. SDS-PAGE fibrinaytography of PS50 and SPS50.

The above results supported the conclusion that the sulfate groups were successfully introduced.

3.2. Biological properties analysis

The SPS50 at different concentrations exhibited weak ability to suppress the superoxide radical (Fig. 5a). It showed the best inhibition result on superoxide radical was 12.12% when the concentration of SPS50 was 2.5 mg/mL.

In this work, free-radical scavenging effect of the SPS50 samples at different concentration on DPPH· was measured. The DPPH· free-radical scavenging capacity of SPS50 was irregular. When the concentration of SPS50 reached 3 mg/mL, the best inhibition (30.03%) was achieved (Fig. 5b).

The scavenging activity of SPS50 against the hydroxyl radical was not significant. At the concentration of 3.0 mg/mL, the highest inhibition (25.07%) was afforded (Fig. 5c).

The free-radical scavenging capacity of SPS50 was lower than that of PS50.

The growth inhibitory effects of SPS50 against K562 cells were first examined. After 24 h co-culture on K562, as shown in Fig. 6a, the PS50 exhibited a high activity in low concentration (25 μg/mL) and high concentration (400 μg/mL). But in the concentration range of 50–200 μg/mL, PS50 demonstrated a low inhibition of K562 cells. Results demonstrated that the growth inhibitory effects of SPS50 were significantly better than PS50, but the effectiveness was irregular. When the concentration of SPS50 reached 100 μg/mL, the inhibition ratio increased to 47.5%.

After treatment for 48 h, as shown in Fig. 6b, PS50 had the same phenomenon as that of 24 h. But SPS50 demonstrated distinct inhibition effect at the concentrations ranging from 50 to 400 μg/mL, whereas the growth inhibitory effects for 24 h treatment were not significant. And the results also showed that SPS50 inhibited K562 cells growth in a dose-dependent manner.

Cell K562 was treated with PS50 and SPS50 for 48 h, then it was collected for the whole body protein analysis. The result was shown in Fig. 7. K562, after 48 h treated with SPS50 (400 μg/mL), the whole protein inside the cell changed notably. Compared with the SP50, the protein in 70 kDa–75 kDa were significantly increased, and the protein in 35 kDa–40 kDa and 26 kDa disappeared. Cell apoptosis is one of the basic biological phenomenon (Zhao & Shang, 2006). After SPS50 treatment, the protein of K562 cell changed dramatically. SPS50 might inhibited or promoted cell apoptosis in gene expression level anomalies. We had another speculation that SPS50 polysaccharide may increase apoptosis of K562 cells. All these hypothesize still need further confirmation.

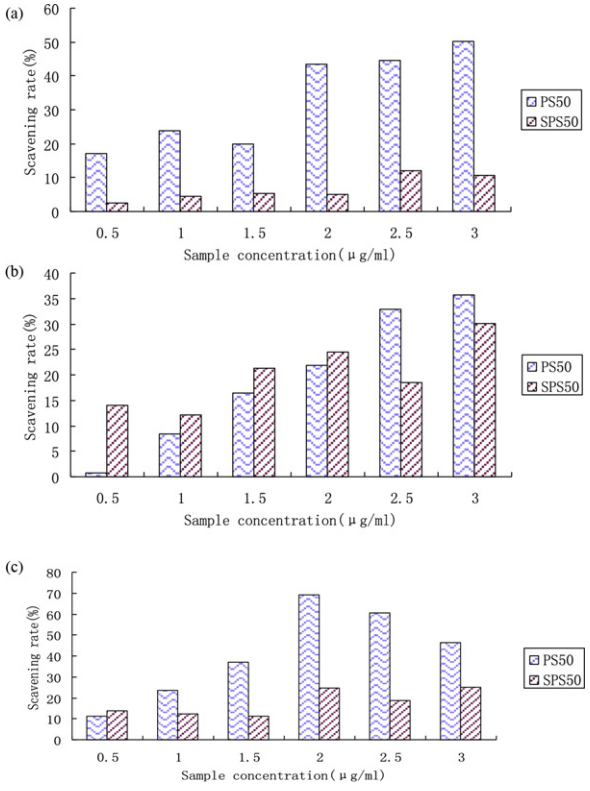

Fig. 5. Scavenging ability of polysaccharides on O^{2-} redical (a), DPPH· radical (b) and ·OH radical (c).

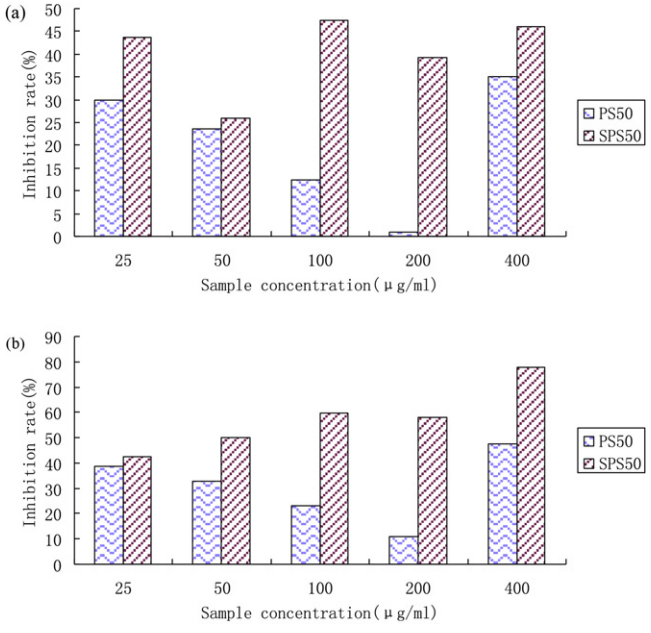

Fig. 6. Inhibition effects of K562 cells incubated with polysaccharides for 24 h (a) and 48 h (b).

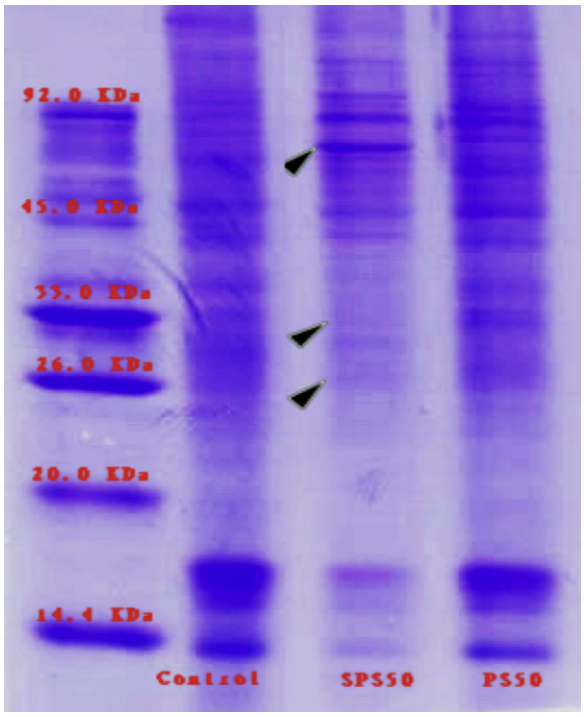

Fig. 7. SDS-PAGE profile of K562 cells incubated with polysaccharides for 48 h.

4. Conclusion

The SPS50 was derived from water soluble polysaccharide of *C. gunnii*. The results showed that the anti-oxidizing capacity of SPS50 was not as significant as P50 and the anti-tumor activity of SPS50 was much better than PS50. SPS50 has shown strong growth inhibition on K562 cells. The tumor inhibition ratio of SPS50 against K562 was 69.92%. This work may provide new directions for the development and application of polysaccharides from *C. gunnii*.

Acknowledgments

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