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International Journal of Biological Macromolecules 39 (2006) 228–233

INTERNATIONAL JOURNAL OF
Biological
Macromolecules
STRUCTURE, FUNCTION AND INTERACTIONS

www.elsevier.com/locate/ijbiomac

Preparation of a chemically sulfated polysaccharide derived from *Grifola frondosa* and its potential biological activities

Xiaohua Nie [a], Baojun Shi [b], Yuting Ding [a], Wenyi Tao [c,*]

[a] *College of Biological and Environmental Engineering, Zhejiang University of Technology, Hangzhou 310014, PR China*
[b] *Guangdong VTR BIO-TECH Co. Ltd., Zhuhai 519060, PR China*
[c] *College of Biotechnology, Southern Yangtze University, Wuxi 214036, PR China*

Received 17 February 2006; received in revised form 26 March 2006; accepted 28 March 2006
Available online 7 April 2006

Abstract

This report describes the preparation, characterization and potential biological activities of a chemically sulfated polysaccharide (S-GAP-P), which was derived from water-insoluble polysaccharide of *Grifola frondosa* mycelia. S-GAP-P was determined to be a glucan sulfate with the average molecular weight of 28 kDa and the sulfur content of 16.4%. The antitumor and immunomodulating activities of the sulfated derivative were estimated in vitro and in vivo. S-GAP-P inhibited the proliferation of SGC-7901 cells and induced apoptosis, in a dose-dependent manner. And the results from in vivo experiments demonstrated that S-GAP-P significantly inhibited the tumor growth and enhanced the peritoneal macrophages phagocytosis in S180-bearing mice. It is noteworthy that S-GAP-P could accelerate the antitumor activity of CTX and improve the immunocompetence damaged by CTX, suggesting the combination might increase cytotoxic efficacy and decrease toxicity of some chemotherapeutic agents in cancer treatment.
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Keywords: Sulfated polysaccharide; *Grifola frondosa*; Characterization; Biological activities; Combination

1. Introduction

In the last decades, much attention has focused on the biological properties of polysaccharides and their chemical derivatives, especially sulfated derivatives [1–4]. Sulfated polysaccharides have a broad range of important bioactivities comprising antioxidant [5], anticoagulant [2] and antithrombotic activities [6]. They are also known to increase the resistance to some virus [7] and inhibit some tumor development [8,9]. Moreover, it is notable that water-insoluble polysaccharides show little bioactivity, whereas such sulfated derivatives exhibit higher water solubility along with bioactivities [10]. The sulfation of polysaccharides should not only enhance the water solubility but also change the chain conformation, resulting in the alteration of their biological activities [11]. Chemical modification of polysaccharides provided an opportunity to obtain new pharmacological agents with possible therapeutic uses.

Grifola frondosa, a traditional and edible mushroom in Southeast Asia, is a Basidiomycete fungus belonging to the order *Aphyllopherales* and family *Polyporeceae*. Extensive studies have demonstrated that *G. frondosa* exhibits considerable physiological activities [1,12,13]. Fruit body and liquid-cultured mycelium of this mushroom have been reported to contain some useful antitumor polysaccharides, and these polysaccharides have been identified as glucans [14,15]. However, there are few investigations about the water-insoluble polysaccharides from *G. frondosa*.

In this paper, for the first time, we prepared the sulfated derivative of water-insoluble polysaccharides alkali-extracted from *G. frondosa* mycelia by chlorosulfonic acid/pyridine method, and tested its potential biological activities in vitro and in vivo. In addition, the effects of the sulfated polysaccharide in combination with cyclophosphamide, a well-known anticancer chemotherapeutic agent, were also investigated.

* Corresponding author. Tel.: +86 571 88320796; fax: +86 571 88320796.
E-mail address: niexiaohua2000@126.com (X. Nie).

0141-8130/$ – see front matter © 2006 Published by Elsevier B.V.
doi:10.1016/j.ijbiomac.2006.03.030

2. Materials and methods

2.1. Microorganism

The strain of *G. frondosa* GF9801 is maintained in Biopharmaceutics Laboratory of Southern Yangtze University (Wuxi, China). The stock culture was maintained on potato dextrose agar (PDA) slants and subcultured every 2 months. The slants were incubated at 25 °C for 7 days and then stored at 4 °C.

2.2. Extraction of water-insoluble polysaccharides

The mycelia of *G. frondosa* GF9801 were obtained through submerged cultivation [16] and repeatedly extracted with distilled water at 80 °C till the detection was negative by the phenol–sulfuric acid method [17], then the mycelia residue was treated with 2% NaOH solution at 4 °C overnight. The extraction was neutralized with acetic acid, and the resulting precipitate was washed with distilled water and resolved in 0.05 mol/L NaOH solution. After centrifugation, the polysaccharides precipitated by 95% ethanol, after washed sequentially with ethanol, acetone and ether, were dried in vacuum to yield the crude polysaccharides named GAP-P, which was subjected to chemical sulfation.

2.3. Preparation and isolation of S-GAP-P

The sulfation regent was prepared using dry pyridine and chlorosulfonic acid as described elsewhere [18]. Briefly, GAP-P sample (200 mg) was suspended in dry formanide (20 mL), and the sulfation reagent was added drop by drop. The mixture was maintained at 45 °C for 6 h with continuous stirring. After the reaction was finished, the mixture was cooled, neutralized with 2.5 mol/L NaOH solution and dialyzed against distilled water. The dialyzate was concentrated under vacuum and precipitated with 95% ethanol. The precipitate was dissolved in distilled water and centrifugated at 12,000 rpm, and the supernatant was loaded on to a column (2.6 cm × 13 cm) of DEAE Sepharose Fast Flow, followed by eluting at a flow rate of 0.25 mL/min successively with distilled water and a gradient of 0–2.0 mol/L NaCl solution. The fractions were combined according to the total carbohydrate content quantified by the phenol–sulfuric acid method. Materials recovered from the main peak was concentrated and lyophilized to give a sulfated polysaccharide coded as S-GAP-P.

2.4. Characterizations of S-GAP-P

The sulfated content (S%) in the sulfated derivate was tested colorimetrically by Antonopoulos' method [19].

Homogeneity and average molecular weight of S-GAP-P were determined by HPGPC method. S-GAP-P aqueous solution (20 mg/mL) run on a Waters apparatus (Waters, USA) equipped with Ultrahydrogel liner column (7.8 mm × 300 mm). The detailed experimental conditions are just as below: column temperature 45 °C; injection volume 20 μL; mobile phase 0.1 mol/L sodium nitrate aqueous solution; mobile rate 0.9 mL/min. And the homogeneity was also tested by agarose gel electrophoresis analysis as previously described [20].

The monosaccharide composition of S-GAP-P was determined by GC chromatography. Completed hydrolysis of S-GAP-P (50 mg) was carried out with 1.0 mol/L H_2SO_4 (2 mL) for 6 h at 100 °C, then the hydrolyzate was neutralized with $BaCO_3$ and evaporated to residue, followed by successive reduction with $NaBH_4$ at 90 °C for 30 min and acetylation with acetic anhydride at 90 °C for 30 min. The resulting product was concentrated to residue and dissolved in chloroform (0.5 mL) for GC analysis. Gas chromatography (GC) of the resulting alditol acetate derivatives was carried out on a GC-14A chromatogram (Shimadzu, Japan) using a quartz capillary column (0.32 mm × 30 m) packed with 3% OV-1701 on Chromosorb WAW DMCS (80–100 mesh) at temperatures programmed from 180 to 240 °C. The flow rate of carrier gas (nitrogen) was maintained at 1.5 mL/min and the injected volume was 10 μL. The fractions were detected by flame ionization detector (FID). The following monosaccharides were used as references: D-rhamnose, L-fucose, D-arabinose, D-xylose, D-mannose, L-glucose, D-galactose, inositol.

Fourier-transformed infrared spectrum was recorded with potassium bromide pellets on a Necolit 5D×B FT-IR spectrophotometer (Necolit, USA) in the range of 400–4000 cm^{-1}.

2.5. Animals and cells

Kuming mice, weighing 20.0 ± 2.0 g, were purchased from the Animal Research Center, Center for Disease Control and Prevention of Jiangsu Province (Wuxi, China). The mice were randomly divided into groups ($n = 10$), and allowed free access to standard laboratory diet and water.

Human gastric carcinoma SGC-7901 cells and mouse sarcoma 180 cells (S180) were obtained from Shanghai Institute of Cell Biology, Chinese Academy of Science (Shanghai, China). The cells were maintained in RPMI1640 medium (Gibco-BRL, USA) supplemented with 10% fetal bovine serum (Gibco-BRL, USA), 1 mmol/L L-glutamine, 50 U/mL penicillin and 50 U/mL streptomycin, and cultured at 37 °C in a humidified 5% CO_2 incubator.

2.6. Assay of cell proliferation

The cell proliferation was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cellular viability assay method [21]. Briefly, exponentially growing cells were plated into 96-well microplates at a density of 1×10^4 cells/well in RPMI1640 medium and incubated for 24 h prior to treatment. The cells were treated with various concentrations of S-GAP-P. At the end of treatment, MTT solution (Sigma, USA) was added to each well and incubated for another 4 h. The medium was removed and 150 μL dimethysulfoxide was added to the wells. Then the absorbance was measured at 570 nm using an ELISA reader (Bio-Tek EL, USA). All determinations were conducted in triplicate. The effect of S-GAP-P on cell proliferation was assessed as percent cell viability.

2.7. Flow cytometric analysis

Apoptotic cells induced were detected by flow cytometry using propidium iodide [22]. SGC-7901 cells were cultured for 48 h in the presence of S-GAP-P. 1×10^6 cells were harvested and fixed in 70% ice-cold ethanol, then stored at −20 °C for 24 h. After centrifugation, the cells were suspended in 1 mL phosphate buffered saline (PBS, 0.01 mol/L, pH7.2) containing 0.1 mg/mL RNase A (Sigma, USA)and 0.1 mg/mL propidium iodide (PI; Sigma, USA), and incubated for 30 min in the dark. The cell suspensions were filtrated and analyzed by a FACScan flow cytometry system (Becton Dickinson, CA, USA). Data from 10,000 cells of each sample were collected for evaluation. Sub-G_0/G_1 DNA content was taken as basis for quantitative evaluation of apoptotic content.

2.8. Antitumor activity in vivo

Six-day-old S180 ascites tumor cells (about 2×10^6 cells; 0.2 mL) were subcutaneously implanted into the right hind groin of the mice. One day after inoculation, the mice were treated with different doses of S-GAP-P and/or cyclophosphamide (CTX) by intraperitoneal injection. The controls were given with physiological saline instead of the test solution. All treatments were administered for 10 days. On day 11, the mice were sacrificed and the tumors were weighed. The inhibitory rate was calculated as follows: inhibitory rate (%) = $[(A - B)/A] \times 100$, where A is the mean tumor weight of the control group, and B is that of the treated group.

2.9. Assay of peritoneal macrophage phagocytosis

Yeast was suspended in isotonic saline, heated for 10 min at 100 °C to remove the impurity, and dried at 70 °C. Then the dried yeast (1 g) was suspended in 100 mL of isotonic saline before the experiment.

After the treatment with S-GAP-P and/or CTX for 10 days, the mice were intraperitoneally injected with 3 mL sterile isotonic saline before sacrifice on day 11. Then ascites (1 mL) was drawn out 2 min later, seeded on a microscope slide, and incubated for 50 min at 37 °C to allow macrophage adhesion. Non-adherent cells were washed away with isotonic saline. The remaining adherent cells were used as monolayer of peritoneal macrophages. The monolayer of peritoneal macrophages was co-incubated with yeast suspension for 40 min at 37 °C. The macrophages were rinsed with isotonic saline to remove non-cell-ingested yeast, fixed with glutaraldehyde fixative, and stained with 1% methylene blue solution (Sigma, USA). For each microscope slide, about 100 macrophages were examined with an Olympus microscope using a 400× objective lens. The phagocytic rate was calculated as the percentage of macrophages with ingested yeast. The phagocytic index was calculated as the mean number of phagocytosed yeast per macrophage [23]. All determinations were conducted in triplicate.

2.10. Statistical analysis

The data obtained were analyzed statistically by ANOVA method. Significance of any differences between groups was evaluated using Student' t-test. All data were expressed as mean ± S.D.

3. Results and discussion

3.1. Characterization of S-GAP-P

The sulfated derivative of water-insoluble polysaccharides produced by *G. frondosa* was prepared according to the procedures of Yoshida et al. [18], and isolated by DEAE Sepharose Fast Flow chromatography (Fig. 1). The main fraction was collected and lyophilized to give a homogenous sulfated polysaccharide (S-GAP-P). The sulfur content in S-GAP-P was 16.4%, indicating that the degree of substitution (DS) was 1.74.

S-GAP-P showed a symmetrical narrow peak on HPGCP, its average molecular weight was determined to be 28 kDa in reference to standard T-Dextran. And the purity of S-GAP-P was further confirmed by agarose gel electrophoresis. As illustrated in Fig. 2, a single and homogeneous band was found on the agarose gel, which was in accordance with previously report [5]. In addition, quantitative determination of monosaccharide composition pattern by GC analysis suggested that S-GAP-P only comprises glucose, which means that S-GAP-P is a glucan sulfate.

In comparison with GAP-P, two characteristic absorption bands appeared in the FT-IR spectrum of S-GAP-P (Fig. 3), one at 1232 cm^{-1} describing an asymmetrical S=O stretching vibration and the other at 810 cm^{-1} representing a symmetrical C–O–S vibration associated with a C–O–SO_3 group, indicating incorporation of the sulfating group [24]. These results indicated that the sulfation reaction had actually occurred.

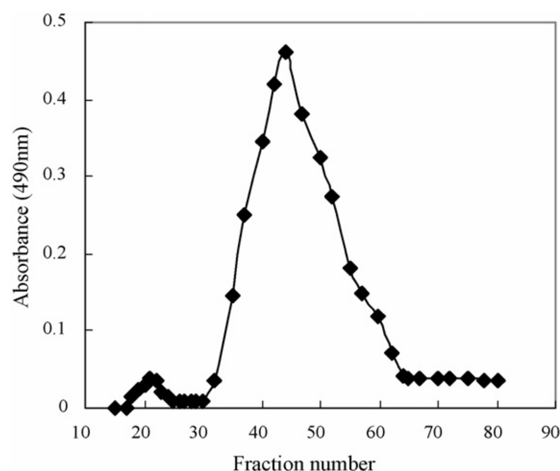

Fig. 1. DEAE Sepharose Fast Flow chromatographic profile for sulfated derivative of water-insoluble polysaccharide extracted from *G. frondosa*.

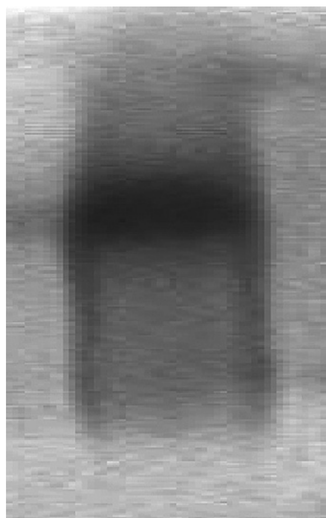

Fig. 2. Agarose gel electrophoresis of S-GAP-P. S-GAP-P solution (10 mg/mL) was applied to a 1% agarose gel and run for 1.5 h at 150 V in 0.06 mol/L barbital buffer (pH 8.6). After electrophoresis, the polysaccharide was stained with 0.1% toluidine blue in AcOH–EtOH–water (0.1:5:5, v/v).

3.2. Effect of S-GAP-P on cell proliferation and apoptosis

A literature survey suggested that chemical sulfation of water-insoluble polysaccharides might lead to antitumor activity [25]. Introduction of sulfate groups to polysaccharide should change its physicochemical characterization and chain conformation, and the forces among sulfate groups result in a relatively expanded and stiff chain in aqueous solution. It was concluded that relatively high chain stiffness and good water solubility of sulfated polysaccharides are beneficial to the enhancement of their antitumor effect [10,26].

In our experiment, we evaluated the anti-proliferative and apoptotic effects of S-GAP-P against human gastric carcinoma SGC-7901 cells and mouse sarcoma 180 cells in vitro. As shown in Fig. 4, S-GAP-P exerted distinct inhibition on SGC-7901 cell proliferation in a dose-dependent manner, and the IC50 for 48 h treatment was 264.28 μg/mL. After treatment for 48 h, S-GAP-P induced accumulation of SGC-7901 cells in sub-G_0/G_1 phrase in a dose-dependent manner, and the apoptotic contents in SGC-7901 cells exposed to 10, 20 and 100 μg/mL S-GAP-P were 2.1%, 6.8% and 13.9%, respectively (Table 1). However, in our experiment, S-GAP-P showed few effects on S180 cells proliferation at the concentrations ranging from 0.1 to 1.5 mg/mL (data not shown).

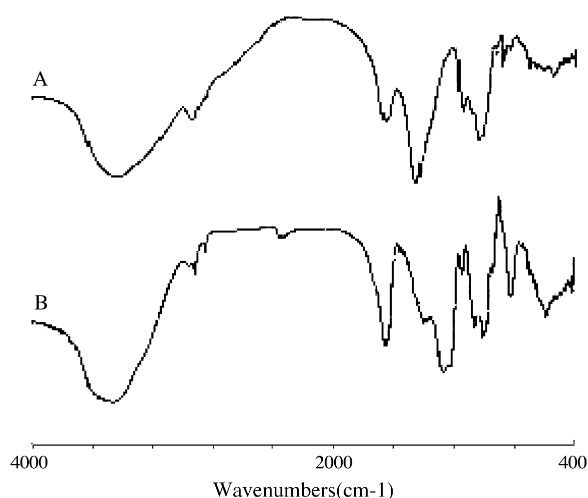

Fig. 3. Comparison of GAP-P and S-GAP-P IR spectra. (A) GAP-P; (B) S-GAP-P.

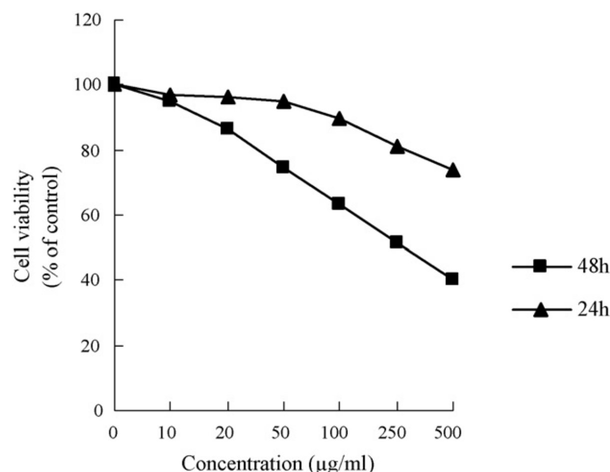

Fig. 4. Growth inhibition of S-GAP-P on human gastric carcinoma cells. SGC-7901 cells were treated with various concentrations of S-GAP-P for 24 or 48 h, and the cell proliferation was determined by MTT assay. The data were expressed as mean ± S.D. of triplicate experiments. Significant differences from the control were evaluated using Student's *t*-test: $^{*}p < 0.05$, $^{**}p < 0.01$.

3.3. Antitumor activity of S-GAP-P and its combinations in vivo

The effects of S-GAP-P and its combinations with cyclophosphamide (CTX) against S180 tumor in mice are summarized in Table 2. S-GAP-P was found to be associated with significant inhibition of S180 tumor growth in a dose-dependent manner, compared with the control group. And the inhibition of S-GAP-P at 50 mg/kg/day dose (55.22%) was slightly lower than that of CTX at 25 mg/kg/day dose (63.15%).

In addition, we tested the combined effects of S-GAP-P and CTX on tumor growth in S180-bearing mice. CTX is a widely used chemotherapeutic drug in cancer treatment that has a broad spectrum of activity against various human tumor [27,28], however, its application is limited because of the high-dose hematopoietic, renal and cardiac toxicity [29]. From the

Table 1
Effect of S-GAP-P on SGC-7901 cells apoptosis

Treatment	Apoptotic content (%)
Control	1.2 ± 0.05
S-GAP-P (10 μg/mL)	2.1 ± 0.16
S-GAP-P (20 μg/mL)	6.8 ± 0.52*
S-GAP-P (100 μg/mL)	13.9 ± 0.71**

Results are expressed as mean ± S.D. ($n = 10$).
* $p < 0.05$ (Student's *t*-test).
** $p < 0.01$ (Student's *t*-test).

Table 2
Inhibitory effects of S-GAP-P and its combinations on S180 tumor growth in vivo

Treatment	Inhibitory rate (%)
S-GAP-P (5 mg/kg/day)	17.46
S-GAP-P (20 mg/kg/day)	43.61**
S-GAP-P (50 mg/kg/day)	55.22**
CTX (10 mg/kg/day)	26.46
CTX (25 mg/kg/day)	63.15**
S-GAP-P (5 mg/kg/day) + CTX (10 mg/kg/day)	55.43**
S-GAP-P (20 mg/kg/day) + CTX (10 mg/kg/day)	67.95**

Results are expressed as mean ± S.D. ($n = 10$); * $p < 0.05$ (Student's t-test).
** $p < 0.01$ (Student's t-test).

results (Table 2), the inhibitory effects of CTX in combination with S-GAP-P were increased from 26.46% to 55.43% and 67.95%, respectively. A significant synergistic interaction was found in the combination of S-GAP-P (5 mg/kg/day) and CTX (10 mg/kg/day), and the interaction was sub-additive between S-GAP-P (20 mg/kg/day) and CTX (10 mg/kg/day).

The in vivo results demonstrated that the polysaccharide sulfate exerted significant antitumor activity and could enhance the cytotoxic efficacy of CTX. The lower dose CTX-containing regimens showed favorable safety-efficacy profiles compared with the standard drug. We presumed that antitumor activity of S-GAP-P might result not only from the direct cytotoxicity but also from the stimulation of the immunoresponse mechanism of the host. Some polysaccharides have been confirmed to have immunomodulating activity, which contribute to restore the host immunity suppressed by tumor [30,31]. The results of the present study was in accordance with previously reports [32,33]. The use of polysaccharides as an adjuvant drug in combination with chemotherapeutic agents may potentially represent an interesting approach for cancer treatment.

3.4. *Effects of S-GAP-P and its combinations on macrophage phagocytosis*

Macrophages have been shown to be important components of the host defense against bacterial infections and tumor cells [34]. They exert diverse functions including phagocytosis, tumor cytotoxicity, cytokine secretion and antigen presentation [35]. Recent reports have demonstrated the activation of macrophage by sulfated polysaccharide [36]. The same result was also found in our experiments (Table 3), the treatment with S-GAP-P resulted in a significant enhancement of peritoneal macrophages phagocytosis in S180-bearing mice. The phagocytic rates and indexes in the S-GAP-P-treated groups were 1.2–1.9-fold and 2.7–5.4-fold those of the control group, respectively. Moreover, the phagocytosis of murine peritoneal macrophage in co-treated groups was distinctly improved compared with those in CTX-treated group (Table 3), the immune function damaged by CTX was presumed to be significantly resumed by S-GAP-P. The results indicated that S-GAP-P could activate peritoneal macrophage and increase the macrophage phagocytosis in a tumor-bearing mouse model.

Table 3
Effects of S-GAP-P and its combinations on macrophage phagocytosis in vivo

Treatment	Phagocytic rate (%)	Phagocytic index
Control	32.42 ± 3.49	0.83 ± 0.25
S-GAP-P (5 mg/kg/day)	40.24 ± 8.51	2.26 ± 0.79*
S-GAP-P (20 mg/kg/day)	59.04 ± 5.44**	4.08 ± 1.37**
S-GAP-P (50 mg/kg/day)	63.79 ± 6.03**	4.50 ± 1.14**
CTX (10 mg/kg/day)	25.58 ± 7.19	0.67 ± 0.30
CTX (25 mg/kg/day)	17.22 ± 6.83*	0.55 ± 0.18
S-GAP-P (5 mg/kg/day) + CTX (10 mg/kg/day)	31.32 ± 2.75	1.59 ± 0.41
S-GAP-P (20 mg/kg/day) + CTX (10 mg/kg/day)	42.56 ± 6.02*	3.43 ± 0.57**

Results are expressed as mean ± S.D. ($n = 10$).
* $p < 0.05$ (Student's t-test).
** $p < 0.01$ (Student's t-test).

4. Conclusion

The sulfated derivate (S-GAP-P) of water-insoluble polysaccharide produced by *G. frondosa* was prepared using the chlorosulfonic acid/pyridine method and isolated by DEAE Sepharose Fast Flow chromatography. S-GAP-P was determined to be a homogeneous glucan sulfate with the average molecular weight of 28 kDa and the sulfur content of 16.4%. Two characteristic absorption bands (1232 and 810 cm^{-1}) appeared in FT-IR spectrum of S-GAP-P, which indicated that the sulfation reaction had actually occurred. S-GAP-P exhibited distinct anti-proliferative effects on SGC-7901 cells and induced apoptosis in a dose-dependent manner, but no effects on S180 cells. The in vivo experiments showed that S-GAP-P significantly inhibited the tumor growth and enhanced the macrophages phagocytosis in S180-bearing mice. It is noteworthy that S-GAP-P could accelerate the antitumor activity of CTX and improve the immunocompetence damaged by CTX.

Acknowledgements

We thank Prof. Jian Jin, Jiali Zhang, Jun Dai, Xiaohong Gu (Southern Yangzi University, Wuxi, China) for their technical assistance and advice.

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