Sulfated modification of the water-soluble polysaccharides from *Polyporus albicans* mycelia and its potential biological activities

Yongxu Sun [a],*, Haitao Liang [b], Guangzhi Cai [c], Shuwen Guan [d], Haibin Tong [e], Xiudong Yang [f], Jicheng Liu [a],*

[a] *Department of Pharmacology, Qiqihar Medical University, Qiqihar 161042, China*
[b] *Transplantation Biology Research Division, State Key Laboratory of Biomembrane and Membrane Biotechnology, Institute of Zoology, Chinese Academy of Sciences, Beijing 100101, China*
[c] *College of Pharmacy, Changchun University of Chinese Medicine, Changchun 130117, China*
[d] *College of Life Science, Jinlin University, Changchun 130012, China*
[e] *Life Science Research Center, Beihua University, Jilin 132013, China*
[f] *Department of Chemistry, Pukyong National University, Busan 608-737, Republic of Korea*

ARTICLE INFO

ABSTRACT

In this study, three chemically sulfated polysaccharides (SPAPs) were derived from one water-soluble polysaccharide (PAP) of *Polyporus albicans* mycelia by chlorosulfonic acid-pyridine method. The effects of polysaccharides on the immune function were examined after the mice were intragastrical administrated with polysaccharides at three doses of 100, 200, and 300 mg/kg body weight for 7 days. The results showed that both the lymphocytes proliferation and macrophage function were significantly enhanced by SPAP in all groups along with the increase of the substitution degree and dose ($P < 0.01$). It indicated that SPAP could be a potential immunostimulants used in the food and pharmaceutical industry.

1. Introduction

Nowadays more and more attention was cast on polysaccharide by biochemical and nutritional researchers due to their various biological activities used in health-care food or medicine, especially immunostimulatory and anti-tumor effects [1–4]. Chemical modification of polysaccharides provided an opportunity to obtain new pharmacological agents with possible therapeutic uses. To our knowledge, there are four main factors influenced the bioactivities of polysaccharides, namely the water solubility, weight-average molecular weight (M_w), chain conformation and introduction of suitable ionic groups with appropriate degree of substitution (DS) [5–7]. One water-insoluble polysaccharide from *P. tuber-regium* was modified by sulfate group exhibited relatively higher in vitro anti-tumor activity against human hepatic cancer cell line HepG2 than itself. Water solubility and introduction of sulfate groups were the

main factors in enhancing the antitumor activities [7]. Furthermore, Many sulfated polysaccharides possessed potent antioxidant, anti-coagulant, antithrombotic and antiviral activities [8–11].

In our previous work [12], one immunoregulatory polysaccharide (PAP), with a molecular mass of 3.7×10^4 Da, was obtained from the mycelia of *Polyporus albicans*. Structure features of the purified polysaccharide were investigated by a combination of chemical and instrumental analysis. Preliminary tests showed PAP had potent stimulating effects on murine lymphocyte proliferation, and was a potential adjuvant for vaccine [3].

In this paper, we prepared the sulfated derivatives of PAP by chlorosulfonic acid-pyridine method for the first time, and explored its biological activities for seeking new pharmaceutical products. In addition, we also try to elucidate the structure–activity relationships among the sulfated polysaccharides.

2. Materials and methods

2.1. Materials and chemicals

Chlorosulfonic acid (CSA), pyridine (Pyr), Sepharose CL-6B was purchased from Pharmacia Biotech and DEAE-Sepharose CL-6B

* Corresponding authors. Tel.: +86 452 6731303; fax: +86 452 6727648.
E-mail addresses: yongxusun1978@yahoo.com.cn (Y. Sun), jichengliu@yahoo.cn (J. Liu).

from Amersham Biosciences. Trifluoroacetic acid (TFA) and Me_2SO were purchased from E. Merck. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), 2′,7′-dichlorofluorescin diacetate (DCFH-DA), Concanavalin A (ConA) and lipopolysaccharide (LPS) were from Sigma Chemical Co. Medium RPMI-1640 was purchased from Gibco Invitrogen Co. The RMPI-1640 medium, used for immunological tests, was supplemented with HEPES buffer 10 μmol/mL, penicillin 100 IU/mL, streptomycin 100 μg/mL, 1-glutamine 2 μmol/mL, 2-mercaptoethanol 50 μmol/L and 10% newborn bovine serum, pH 7.2. All other reagents were of grade AR.

2.2. Purification of polysaccharide and sulfated modification

The dried mycelia of *P. albicans*, obtained through submerged cultivation [12], were extracted 3× with 95% EtOH at 75 °C for 5 h under reflux to remove lipid. The residue was then extracted using 10 vol of distilled water at 75 °C for three times and 3 h for each time. After centrifugation (1600 × *g* for 10 min, at 20 °C), the supernatant was concentrated 10-fold, and precipitated with 95% EtOH (1:5, v/v) at 4 °C for 12 h. The precipitate collected by centrifugation was deproteinated by a combination of proteinase and Sevag method [13] and exhaustively dialyzed against water for 2 days. The precipitate by 5 vol of 95% EtOH was collected and freeze dried to obtain the crude polysaccharide, named as CPAP. The CPAP (100 mg) was further purified on a Sepharose CL-6B column (90 cm × 2 cm) eluted with 0.9% sodium chloride at a flow rate of 0.5 mL/min, and the eluted solution was only separated into one fraction. Fractions (test tube nos. 36–40) containing a large amount of sugar were applied to a Sephadex G-25 column to remove salts, and freeze dried to obtain purified polysaccharide (82 mg), named as PAP.

The sulfation regent was prepared in three levels with the ratio of CSA to Pry in 1:10, 1:8 and 1:6, respectively. Briefly [14], PAP (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. Finally, the sulfated polysaccharide was dialyzed against distilled water and freeze-dried to afford samples coded as $SPAP_1$, $SPAP_2$ and $SPAP_3$. A stock SPAP solution was prepared by dissolving in 0.89% saline and sterilized by passing it through a 0.22 μm Millipore filter. Contaminant endotoxin was analyzed by a gel-clot *Limulus* amebocyte lysate assay. The endotoxin level in the SPAP solution was less than 0.5 EU(endotoxin units)/mL.

2.3. Characterizations of SPAP

Total carbohydrate content was measured by the phenol-sulfuric acid method, using d-glucose as the standard [15]. The substitution degree with sulfate was established on the basis of the sulfate content, determined by barium chloride-gelatin assay. The DS (w/w) was calculated according to the equation: $DS = 162 \times (SO_4^2 \ %)/100 - (96/98 \times SO_4^2 \ %)$ [16]. The FT-IR spectra (KBr pellets) were recorded on SPECORD in a range of 400–4000 cm^{-1}. The homogeneity and M_w of PAP and SPAP were determined by high-performance size-exclusion chromatography (HPSEC) [13], which was performed on a SHIMADZU HPLC system fitted with one TSK-G30007 columns (7.8 mmID × 30.0 cmL) and a SHIMADZU RID-10A detector. The data were processed by GPC processing software ($Millennium^{32}$ Version). The mobile phase was 0.7% Na_2SO_4, and the flow rate was 0.5 mL min^{-1} at 40 °C, with 1.6 mPA. The molecular mass was estimated by reference to a calibration curve made from a set of Dextran T-series standards of known molecular mass (T-130 80, 50, 25, and 10).

2.4. Experimental animals and isolation of peritoneal macrophages

Male BALB/c mice (6–8 weeks old) weighing 18–22 g were purchased from the Pharmacology Experimental Center of Jilin University and acclimatized for 1 week prior to use. All mice were housed under standard conditions at 24 ± 1 °C, with relative humidity of 50, and a 12/12 h light/dark cycle. The mice were randomly divided into groups (*n* = 13), and rodent laboratory chow pellets and tap water were supplied ad libitum. All mice (*n* = 65) were forcedly feeding with polysaccharides (PAP, $SPAP_1$, $SPAP_2$ or $SPAP_3$) using a stomach tube at various doses of 100, 200 and 400 mg/kg body weight, respectively. Saline-treated animals were included as controls.

After treated with PAP or SPAP for 7 days, the mice were sacrificed and peritoneal exudate cells were collected by lavage with 10 mL of Hank's balanced salt solution as described previously [17]. The collected cells were seeded and cultured in RPMI-1640 at a density 2×10^6 cells/well. The cells were allowed to adhere for 2 h to a 96-well culture plate at 37 °C with a 5% CO_2 humidified incubator. After a 2-h incubation, nonadherent cells were removed by washing with the medium three times and the adhered macrophages were cultured for another 24 h.

2.5. Splenocyte proliferation assay in vivo

Spleen cells of mice were prepared by gently mincing and grinding the spleen fragment in RPMI-1640 medium on a fine steel mesh. To isolate mononuclear cells, 5 mL aliquots of the spleen cell suspension were layered onto 2.5 mL aliquots of a polysucrose–sodium ditrizoate solution and centrifuged at 300 × *g* at 4 °C for 10 min. Mononuclear cells were gently removed from the interface between medium and histopaque and transferred to a sterile container and washed with RPMI-1640. At last, the cells were resuspended in 5 mL RPMI-1640 medium, and cell numbers were done with a haemocytometer by trypan blue dye exclusion technique. Cell viability exceeded 95%. Splenocyte proliferation was assayed as previous described [18]. Briefly, An aliquot of 100 μL of splenocytes at 5×10^6 cells/mL was seeded into each well of a 96-well flat-bottom microtiter plate, thereafter ConA (final concentration 5.0 μg/mL), LPS (final concentration 10.0 μg/mL), or medium were added giving a final volume of 200 μL. After preincubation for 68 h at 37 °C in a humidified 5% CO_2 incubator, 50.0 μL of 0.4% MTT was added into each well. The plate was incubated for another 4 h and centrifuged (1400 × *g*, 5 min) to removed the untransformed MTT carefully by pipetting. Then to each well a total of 200 μL DMSO was added to fully dissolve the colored material. The absorbance at 570 nm with a 630 nm reference was measured on an ELISA reader (Model 680, Bio-Rad Instruments). Each experiment was performed in triplicate.

2.6. Analysis of nitric oxide (NO) and reactive oxygen species (ROS) production

Cells were incubated at 37 °C in the presence of 5% CO_2 for 24 h and 100 μL of the cell culture supernatants were removed and analyzed for NO using a colorimetric method with $NaNO_2$ as the standard [19]. Briefly, supernatants were mixed with an equal volume of Griess reagent (1% sulfanilamide, 0.1% *N*-1-naphthylethylenediamine dihydrochloride and 5% phosphoric acid) at room temperature for 20 min. Absorbance was read at 540 nm and results were shown as μmol/mL.

ROS levels were determined by measuring the oxidative conversion of the sensitive fluorescent probe 2′,7′-dichlorofluorescein diacetate (DCFH-DA) to fluorescent 2′,7′-dichlorofluorescein (DCF)

Table 1
The carbohydrate content, DS and M_w of polysaccharides, and their effects on ConA- or LPS-induced proliferation activity of mouse splenocytes in vivo[a].

Sample	Dose (mg/kg)	ConA (SI)	LPS (SI)	Carbohydrate content (%)	DS (%)	M_w (Da)
Control	–	0.35 ± 0.04	0.32 ± 0.03	–	–	–
PAP	100	0.45 ± 0.05[c]	0.41 ± 0.04[b]			
	200	0.50 ± 0.06[c]	0.46 ± 0.03[c]	94.56	–	3.7×10^4
	400	0.53 ± 0.07[c]	0.49 ± 0.06[c]			
SPAP$_1$	100	0.46 ± 0.04[c]	0.44 ± 0.04[c]			
	200	0.52 ± 0.03[c]	0.53 ± 0.04[c]	65.49	0.46	4.7×10^4
	400	0.59 ± 0.02[c]	0.58 ± 0.07[c]			
SPAP$_2$	100	0.45 ± 0.06[c]	0.44 ± 0.05[c]			
	200	0.55 ± 0.07[c]	0.56 ± 0.04[c]	50.04	0.56	5.6×10^4
	400	0.61 ± 0.03[c]	0.60 ± 0.04[c]			
SPAP$_3$	100	0.45 ± 0.07[c]	0.48 ± 0.06[c]			
	200	0.57 ± 0.02[c]	0.55 ± 0.04[c]	46.77	0.78	7.0×10^4
	400	0.64 ± 0.04[c]	0.69 ± 0.02[c]			

Purified polysaccharide was named as PAP. Sulfated polysaccharide was named as SPAP.
[a] The values are presented as mean ± S.D.
[b] $P < 0.05$, significantly different from the control.
[c] $P < 0.01$, significantly different from the control.

as described by Wang and Joseph [20]. The fluorescence intensity of DCF is proportional to ROS production. At the end of incubation, cells with a concentrations of 2×10^6 cells/well in 96-well plate were washed two times with PBS prior to loading 5 μL DCFH-DA (final concentration 10 μm in DMSO) at 37 °C for 35 min in dark. Then the cells were resuspended with PBS. The fluorescence intensity was recorded at 485 nm excitation and 535 nm emission. Relative ROS production was expressed as a percentage of DCF fluorescence of control.

2.7. Statistical analysis

The data were expressed as mean ± standard errors (S.D.) and examined for their statistical significance of difference with Student's t-test. P-values of less than 0.05 were considered to be statistically significant.

3. Results and discussion

3.1. Characterization of SPAP

Three sulfated derivatives (SPAP$_1$, SPAP$_2$ and SPAP$_3$) were prepared by chlorosulfonic acid-pyridine method. The DS, carbohydrate content and M_w of three polymers were listed in Table 1. The DS of SPAP were listed in a decreasing order as follows: SPAP$_3$ > SPAP$_2$ > SPAP$_1$. However, the carbohydrate content of SPAP listed in a converse order, namely SPAP$_1$ > SPAP$_2$ > SPAP$_3$. Each SPAP showed a single and symmetrically sharp peak, indicating its homogeneity on HPSEC (data not shown), According to the retention time, their molecular weight were estimated to be 7.0×10^4 (SPAP$_3$) Da, 5.6×10^4 (SPAP$_2$) Da and 4.7×10^4 (SPAP$_1$) Da.

In comparison with PAP, two characteristic absorption bands appeared in the FT-IR spectrum of SPAP (data not shown), one at $1232 \pm 3\,cm^{-1}$ describing an asymmetrical S=O stretching vibration and the other at $810 \pm 3\,cm^{-1}$ representing a symmetrical C–O–S vibration associated with a C–O–SO$_3$ group, indicating incorporation of the sulfating group [21]. These results indicated that the sulfation reaction had actually occurred.

3.2. Effect of PAP and SPAP on splenocyte proliferation

As shown in Table 1, After exposed to either Con A or LPS, the level of stimulated splenocyte proliferation was significantly

higher in the groups treated with PAP (100, 200 and 400 mg/kg), SPA$_1$ (100, 200 and 400 mg/kg), SPA$_2$ (100, 200 and 400 mg/kg) and SPA$_3$ (100, 200 and 400 mg/kg) than the control group ($P < 0.05$ or $P < 0.01$). Between groups, the level of splenocyte proliferation was enhanced along with the DS raise of polysaccharides as follow: SPAP$_3$ > SPAP$_2$ > SPAP$_1$ > PAP. Among group, splenocyte proliferation is stimulated by polysaccharides in a dose-dependent manner. An effective T cell-mediated immunity, which can be shown by the stimulation of lymphocyte proliferation response, plays an important role to combat intracellular microbe infections [22]. Among the T lymphocytes, helper T cells induce B-lymphocytes to secrete antibodies. Help T cells can be divided into two subsets of effector cells, namely Th1 and Th2 cells. The Th1 cells secrete cytokines, such as interleukin-2 (IL-2), tumor necrosis factor-β (TNF-β) and interferon-γ (IFN-γ), and stimulate the production of IgG2a, IgG2b and IgG3 in mice. They are responsible for cytotoxic T lymphocyte (CTL) production [23–25]. The findings indicated that PAP and SPAP are potential adjuvants of therapeutic factor against pathogens and cancer.

3.3. Effect of PAP and SPAP on the activation of peritoneal macrophage

The effects of PAP and SPAP on the production of NO by peritoneal macrophages in vivo were shown in Fig. 1. The levels
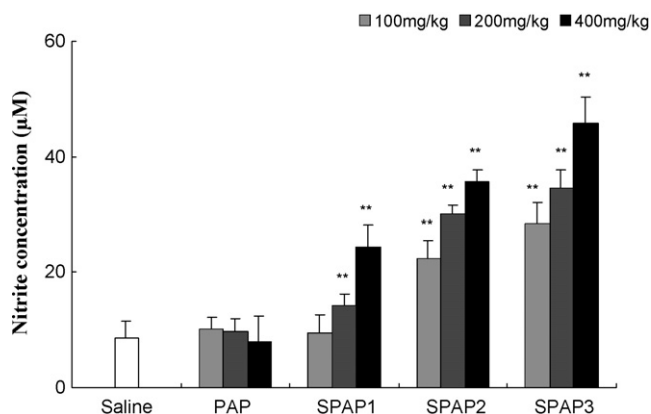
Fig. 1. Effect of polysaccharides on the production of nitrite (NO) by peritoneal macrophages in vivo. The values are presented as mean ± S.D. Significant differences with saline group was designated as **$P < 0.01$.
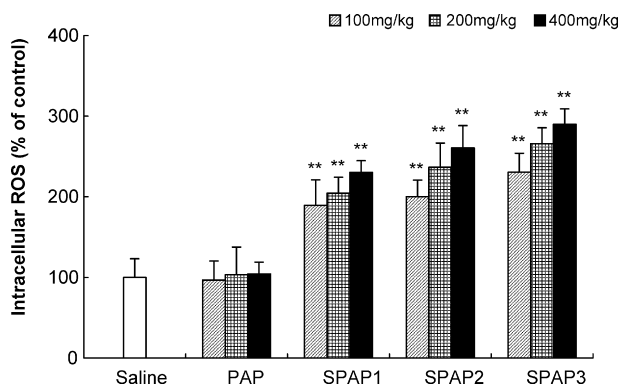
Fig. 2. Effect of polyaccharides on the production of reactive oxygen species (ROS) by peritoneal macrophages in vivo. Relative ROS production was expressed as a percentage of DCF fluorescence of control. The values are presented as mean ± S.D. Significant differences with saline group was designated as **$P < 0.01$.

of NO in all groups treated with SPAP were significantly higher than that in the control group ($P < 0.01$). However, no significant differences were observed between the PAP (100, 200 and 400 mg/kg) groups and the control group ($P > 0.05$). The increase of NO release was accompanied with the raise of DS and dose of SPAP.

The effects of PAP and SPAP on the production of ROS by peritoneal macrophages in vivo were shown in Fig. 2. The PAP (100, 200 and 400 mg/kg) groups did not stimulate the production of ROS ($P > 0.05$), on the other hand, the production of ROS was enhanced by all SPAP groups, compared with the control ($P < 0.01$). Furthermore, SPAP stimulated the production of ROS in a dose-dependent manner.

To the best of our knowledge, macrophages are known to play an essential role in the host defense against primary infection and/or metastatic neoplasia [26]. Macrophages exert their tumoricidal activity not only by direct contact but also by the release of a number of cytotoxic/cytostatic factors such as tumor necrosis factor, interleukin-1, ROS, and reactive nitrogen species (RNS) [27]. Macrophages can be activated to become cytotoxic with a variety of agents that include several cytokines, bacterial-derived LPSs, and various chemotherapeutic drugs [28]. It has been established that NO produced by macrophages is involved in the destruction of various intracellular pathogens as well as tumor cells, and places cells in cytostasis [29]. From the above results it was evident that SPAP strongly induced the activation of macrophage. As a consequence, the higher the DS of polysaccharide, the stronger the activity of macrophage will be.

4. Conclusion

In this study, three sulfated derivates (SPAP) were prepared from the purified water-soluble polysaccharide (PAP) of *P. albicans*

mycelia by chlorosulfonic acid-pyridine method. The DS, carbohydrate content and M_w of three polymers were listed in Table 1. Each SPAP showed a single and symmetrical sharp peak, indicating its homogeneity on HPSEC. Two characteristic absorption bands (1231 and 812 cm^{-1}) appeared in FT-IR spectrum of SPAP, which indicated that the sulfation reaction had actually occurred. Through chemical modification of PAP, we get three optimal biological response modifiers, which can stimulate both proliferation of lymphocytes and the macrophage function in vivo along with the increase of the DS and dose. The results indicated SPAP had potent immunomodulatory properties and could be explored as a potential adjuvant against cancer used in the food and pharmaceutical therapy.

References

[1] X.H. Nie, B.J. Shi, Y.T. Ding, W.Y. Tao, Int. J. Biol. Macromol. 39 (2006) 228–233.
[2] Y. Saima, A.K. Das, K.K. Sarkar, A.K. Sen Sr., P. Sur, Int. J. Biol. Macromol. 27 (2000) 333–335.
[3] Y.X. Sun, J.C. Liu, Vaccine 26 (2008) 3932–3936.
[4] Z. Ruan, J. Su, H.C. Dai, M.C. Wu, Int. Immunopharmacol. 5 (2005) 811–820.
[5] J.H. Yang, Y.M. Du, R.H. Huang, Y.Y. Wan, Y.Y. Li, Int. J. Biol. Macromol. 31 (2002) 55–62.
[6] X.Y. Huang, D.Y. Wang, Y.L. Hu, Y. Lu, Z.H. Guo, X.F. Kong, J.L. Sun, Int. J. Biol. Macromol. 42 (2008) 166–171.
[7] Y.Z. Tao, L.N. Zhang, P.C.K. Cheung, Carbohydr. Res. 341 (2006) 2261–2269.
[8] R. Xing, S. Liu, H.H. Yu, Z.Y. Guo, Z. Li, P.C. Li, Carbohydr. Polym. 61 (2005) 148–154.
[9] C. Urbinati, A. Bugatti, P. Oreste, FEBS Lett. 568 (2004) 171–177.
[10] S. Soeda, Y. Ohmagari, H. Shimeno, A. Nagamatsu, Thromb. Res. 72 (1993) 247–256.
[11] F. Han, W.B. Yao, X.B. Yang, X.N. Liu, X.D. Gao, Int. J. Biol. Macromol. 36 (2005) 201–207.
[12] Y.X. Sun, S.S. Wang, T.B. Li, X. Li, L.L. Jiao, L.P. Zhang, Bioresour. Technol. 99 (2008) 900–904.
[13] Y.X. Sun, J.C. Liu, Int. J. Biol. Macromol. 43 (2008) 279–282.
[14] T. Yoshida, Y. Yasuda, T. Mimura, Y. Kaneko, Carbohydr. Res. 276 (1995) 425–436.
[15] H.B. Tong, Z.Y. Liang, G.Y. Wang, Carbohydr. Polym. 71 (2008) 316–323.
[16] W. Zhang (Ed.), Biochemical Technology of Study on Carbohydrate, Zhejiang University Press, Hangzhou, 1999, pp. 24–93.
[17] G.Y. Kim, G.S. Choi, S.H. Lee, Y.M. Park, J. Ethnopharmacol. 95 (2004) 69–76.
[18] Y.X. Sun, M.Q. Li, J.C. Liu, Int. Immunopharmacol. 8 (2008) 1095–1102.
[19] R. Keller, M. Geiges, R. Keist, Cancer Res. 50 (1990) 1421–1425.
[20] H. Wang, J. Joseph, Free Radic. Biol. Med. 27 (1999) 612–616.
[21] R. Falshaw, R.H. Furneaux, Carbohydr. Res. 307 (1998) 325–331.
[22] A.K. Abbas, A.H. Lichtman (Eds.), Basic Immunology, WB Saunders, Philadelphia, 2004, pp. 1038–1048.
[23] S.L. Constant, K. Bottomly, Annu. Rev. Immunol. 15 (1997) 297–322.
[24] G.F. Del Prete, M. De Carli, M. Ricci, S. Romagnani, J. Exp. Med. 174 (1991) 809–813.
[25] D.F. Fiorentino, M.W. Bond, T.R. Mosmann, J. Exp. Med. 170 (1989) 2081–2095.
[26] N. Lavnikova, L. Burdelya, A. Lakhotia, N. Patel, S. Prokhorova, D.L. Laskin, J. Leukoc. Biol. 61 (1997) 452–458.
[27] F.Y. Liew, Immunol. Lett. 30 (1991) 193–197.
[28] K.A. Foon, Cancer Res. 49 (1989) 1621–1639.
[29] I.J. Fidler, A.J. Schroit, Biochim. Biophys. Acta 948 (1998) 151–173.