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Structural and immunological studies of a major polysaccharide from spores of *Ganoderma lucidum* (Fr.) Karst

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Abstract

A polysaccharide isolated from spores of the fungus, *Ganoderma lucidum*, was found to be a complex glucan. On the basis of compositional and methylation analyses, periodate oxidation, Smith degradation, 1D and 2D NMR, and ESIMS experiments of the native polysaccharide and its degraded products, the polysaccharide was shown to have a backbone of β-(1→3)-linked D-glucopyranosyl residues, with branches of mono-, di- and oligosaccharide side chains substituting at the C-6 of the glucosyl residues in the main chain. Conformational analysis in aqueous solution and immunological activities of the native and degraded glucans were also investigated. The results suggested that the degree of substitution on the main chain and the length of side chains may be very important factors in determining the conformation and the biological activities of β-(1→3)-linked glucans.

Keywords: Fungus; *Ganoderma lucidum*; Spores; Conformational analysis; Immunological activities

1. Introduction

The fungus *Ganoderma lucidum* has been used as a folk medicine in China and has recently attracted much attention on account of its biological activities.[1–3] Miyazaki and Nishijima previously separated a heteroglycan having an antitumor effect from the fruit bodies of the plant.[4] Moreover, Hikino and coworkers isolated several hypoglycemic glycans from another fraction of the same crude polysaccharide.[5,6] However, the polysaccharides in spores of *G. lucidum* have been less studied owing to the difficulty in collection and sporoderm-breaking of the spores. In our previous report,[7] we have described the structure and conformational behavior of a polysaccharide from spores of *G. lucidum*. In continuation of our work on the polysaccharides of the spores of *G. lucidum*, we report herein the structural determination, conformational analysis and immunological assays of a highly branched glucan and its degraded products from sporoderm-broken spores of *G. lucidum*.

2. Results

The native polysaccharide (SP) was eluted as a single symmetrical peak corresponding to

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an average-molecular weight of 1.0×10^4 as determined by the HPSEC method.[8] These data indicated that the polysaccharide was homogeneous. No absorbance at 280 nm and a negative response to the Lowry method showed that the polysaccharide contained no protein.

Sugar compositional analysis of SP, determined by TLC and GLC analysis as the alditol acetates,[9] indicated that it was composed only of D-glucose. The low positive specific rotation, $[\alpha]_D^{21} + 8.22°$ (c 0.82, water) and the characteristic absorption at 890 cm^{-1} in the IR spectrum were indicative of the β-D-glucosidic linkages.

The ^{13}C NMR spectrum of the native polysaccharide (Fig. 1, bottom) was closely similar to that of fungal β-D-glucans.[10] It showed multiple resonances that revealed the structural complexity of the glucan. The β configuration of the D-glucosyl residues was confirmed by the presence of two anomeric peaks in the region of δ 104.4–104.8, and the branching points at C-6 were shown by the signal of the O-substituted carbon atoms at δ 70.9 and the unsubstituted C-6 at δ 62.9. The multiplicity of the signals and the broad C-3 signal in the region at δ 86.2–87.1 could be ascribed to the presence in the glucan of linear β-D-(1→3), branched β-D-(1→3,6) and terminal β-D-glucopyranosyl residues. The presence of the (1→6)-linked residues was evidenced by the low-intensity signal at δ 68.1 and the signal of O-substituted CH_2 at δ 68.1 in a DEPT experiment (data not shown). All these resonances indicated a branched β-D-(1→3)-linked glucan structure of the native polysaccharide.

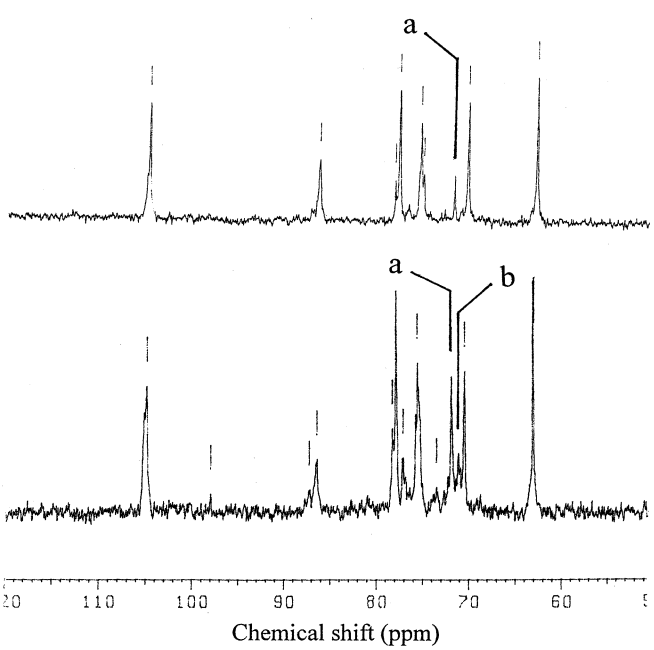


Fig. 1. ^{13}C NMR spectra (100 MHz) of the native polysaccharide (bottom), and Smith-degraded polysaccharide (top) in D_2O at room temperature: (a) C-4 signal of the branched chains; (b) C-6 signal of the (1→3,6)-linked glucosyl residues.

The exact branching of the structure of SP was elucidated by methylation analysis and Smith degradation. The native glucan was methylated by the method of Needs and Selvendran,[11] and the fully methylated polysaccharide was hydrolyzed with acid, reduced and acetylated. GLC–MS analysis revealed the presence of 2,3,4,6-tetra-, 2,4,6-tri-, 2,3,4-tri- and 2,4-di-*O*-methyl-glucitol acetates in the molar ratios of 2:7:0.5:2. These results established that the glucan consisted of (1→3), (1→6), (1→3,6)-linked and nonreducing terminal glucopyranose components. The relatively low proportion of (1→3)-linked residues to the terminal and (1→3,6)-linked residues, together with the low value of the (1→6)-linked glucosyl residues, suggested that the backbone chain of the native glucan was essentially composed of consecutive (1→3)-linked glucopranosyl residues with a highly branched structure.

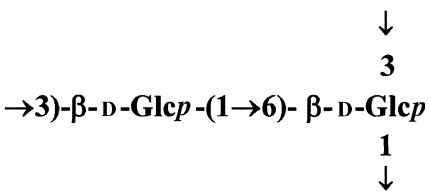
The native polysaccharide was applied to periodate oxidation, sodium borohydride reduction and hydrolysis under mild conditions by heating with 0.2 M TFA at 40 °C for 24 h.[7,12] The Smith-degraded products were dialyzed against distilled water (1000 mL). The nondialysate fraction was also a homogeneous polysaccharide (SP1, molecular weight was 6400) evaluated by HPSEC. The degraded glucan was composed of only glucose monomers. The methylation results revealed that it consisted of 2,3,4,6-tetra-, 2,4,6-tri- and 2,4-di-*O*-methylglucoses in the molar ratios of 2:11:2. This result was in agreement with a (1→3)-linked backbone chain. Since the branching chains still occurred in SP1 after periodate oxidation and Smith degradation of SP, the undestroyed glucose residues in the branchings of SP1 must be a part of (1→3)-linked oligosaccharide side chains in the na-

Table 1
^{1}H and ^{13}C chemical shifts (ppm) of oligosaccharide S-2 obtained after Smith-degradation of SP

	H-1	H-2	H-3	H-4	H-5	H-6	H-6′	C-1	C-2	C-3	C-4	C-5	C-6
Glc (A)	4.73	3.35	3.42	3.40	3.48	3.94 [a]	3.73 [a]	105.5	76.1	78.7	72.3	78.4	63.4
Glc (B)	4.51	3.51	3.77	3.50	3.48	3.96 [a]	3.75 [a]	105.2	75.6	87.0	70.9	78.2	63.4
Gro (C)	3.98/3.69	3.94	3.65/3.60					73.7	73.3	65.0			

[a] The chemical shifts for H-6/H-6′ are degenerate.

tive polysaccharide. These data indicated the presence of the following sequence in the branched backbone chain of the native glucan:



The previous article established that under the aforementioned hydrolysis conditions of mild Smith degradation, the polyhydroxyl groups could be completely removed and not be accompanied by the cleavage of the (1→3) bonds in the backbone chain of this type of glucan.[7] Therefore, the high-molecular weight of the degraded glucan in comparison with that of the native polysaccharide showed the main chain of the native glucan contained no (1→6) linkages. On the other hand, the increased content of the (1→3)-linked glucosyl residues of the Smith-degraded glucan indicated the presence of monosaccharide side chains and (or) short chains of (1→6)-linked glucosyl units in the native polysaccharide. In the ^{13}C NMR spectrum of the degraded glucan (Fig. 1, top), the intensity of the C-4 signal of side chains and the C-6 signal of (1→3,6)-linked glucosidic residues decreased remarkably in comparison with those of the native glucan, suggesting that the degraded glucan had a more linear structure than the native one. These results were in agreement with the methylation analysis.

The dialysate portion of the Smith-degraded products was fractionated by Sephadex G-10 column chromatography. Three fragments were obtained. These were free glycerol, originating principally from terminal glucosyl residues, and two oligosaccharides, S-1 and S-2, composed of glucose and glycerol. S-1 was eluted at a volume between that of monosaccharides and disaccharides. One major residue, terminal D-glucose, was identified by methylation of S-1. The ESIMS spectrum of S-1 contained an ion at m/z 277 that corresponds to $[M + Na]^+$ of an oligosaccharide composed of a hexosyl residue and a glycerol. The monoglycosyl–glycerol in S-1 was identified by its ^{1}H and ^{13}C NMR spectra. The ^{1}H and ^{13}C NMR spectra showed one anomeric proton and carbon signal at δ 4.49 ($^{3}J_{1,2}$ 7.8 Hz) and δ 104.67, respectively (spectra not shown). These results established that the fragment S-1 had the following structure:

β-D-Glc*p*-(1→1)-D-Gro (S-1)

S-2 was eluted at a smaller retention time than that of S-1, indicating that the S-2 molecule was larger than S-1. The retention time observed by gel-filtration chromatography suggested that S-2 was a trisaccharide or disaccharide in comparison with those of the standard sugars. Two major residues, a (1→3)-linked residue and a nonreducing terminal glucose, were identified by methylation analysis. The ESIMS spectrum of S-2 contained an ion at m/z 439 that corresponds to $[M + Na]^+$ of an oligosaccharide composed of two hexosyl residues and a glycerol. The ^{1}H and ^{13}C NMR spectra were totally assigned by HMQC and HMBC (Table 1). The cross peaks in HMBC (Fig. 2), A (1,3) showed that two glucosyl residues connected with (1→3) bond, and B (1,1) signal showed that C-1 of glycerol was linked to C-1 of glucosidic residue. Thus, S2 had the following sequence:

β-D-Glc*p*-(1→3)-β-D-Glc*p*-(1→1)-D-Gro (S-2)
(A) (B) (C)

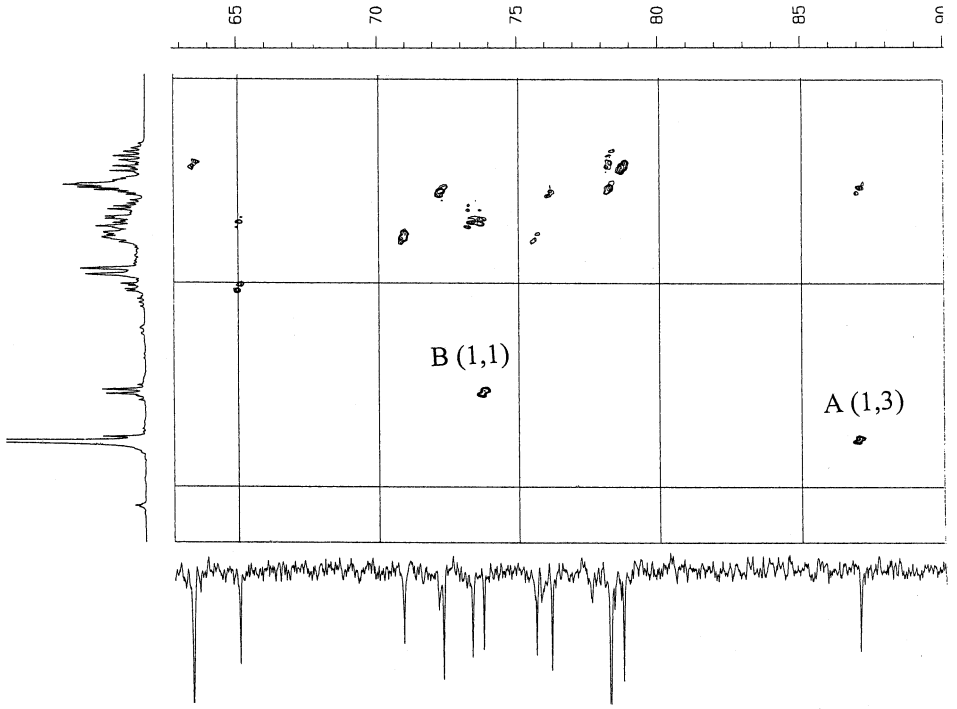


Fig. 2. Partial contour plot of the HMBC spectrum (400 MHz) of the oligosaccharide S-2 obtained after Smith degradation of SP.

These fragments, S-1, S-2 and free glycerol, arose from the cleavage of acetal linkages of the polyhydroxyl groups derived from terminal and *O*-6-glucose residues in:

β-D-Glc*p*-(1→[3)-β-D-Glc*p*-(1→]$_n$6)-β-D-Glc*p*-(1→

n = 0, 1 or 2

A combination of the aforementioned results suggested the native polysaccharide SP probably had the structure shown below (Scheme 1).

To recognize the ordered structures of the native and Smith-degraded glucans, the formation of complexes with Congo Red was investigated.[13] The λ_{max} of Congo Red shifted to a higher wavelength (from 484.2 to 508.4 nm) in the presence of the native glucan, while the λ_{max} of the complex of glucan with Congo Red decreased with the addition of sodium hydroxide (Fig. 3). However, the changes of λ_{max} of Congo Red complex in the presence of the degraded glucan were much different from those of the native glucan, but similar with

R
1
↓
6

[→3)-β-D-Glc*p*-(1→3)-β-D-Glc*p*-(1→]$_m$3)-β-D-Glc*p*-(1→3)-β-D-Glc*p*-(1→]$_m$]$_x$

R = β-D-Glc*p*-(1→

R = β-D-Glc*p*-(1→3)-β-D-Glc*p*-(1→6)-β-D-Glc*p*-(1→

R = β-D-Glc*p*-(1→3)-β-D-Glc*p*-(1→6)-β-D-Glc*p*-(1→3)-β-D-Glc*p*-(1→

n = 0, 1 or 2 2 (n + n') + m + m' = 7

Note: three of the four branching chains are terminal glucosidic residues.

Scheme 1.

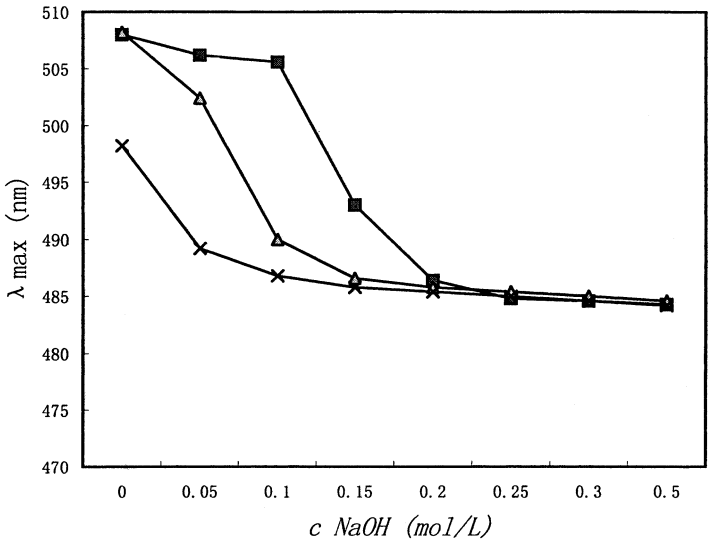


Fig. 3. Changes in the absorption maximum of the Congo Red–polysaccharide complex at various concentrations of alkali. ▲–▲, native polysaccharide; ■–■, degraded polysaccharide; **x**–**x**, control without polysaccharide.

Table 2
Effects of SP and SP-1 [a] on lymphocyte proliferation, antibody production, serum IgG and complement (C-3) levels in mice [b]

	IgG (g/L)	C-3 (g/L)	Hemolysin (A_{520})	Lymphocyte proliferation	
				T cells	B cells
SP	18.9 ± 2.0	2.42 ± 0.12 [c]	1.23 ± 0.06	0.84 ± 0.06 [c]	0.93 ± 0.02 [d]
SP1	19.7 ± 2.3	2.10 ± 0.36	1.21 ± 0.02 [d]	0.95 ± 0.02 [e]	0.94 ± 0.01 [d]
Control	17.3 ± 1.5	2.08 ± 0.35	1.11 ± 0.02	0.55 ± 0.02	0.60 ± 0.04

[a] SP-1 is the Smith-degraded polymer of the native polysaccharide SP.
[b] Results are presented as mean ± S.D. ($n = 7$). The mice were given samples by intraperitoneal injection at a dose of 50 mg/kg for 4 days.
[c] $P < 0.05$, significant difference from the control.
[d] $P < 0.01$, significant difference from the control.
[e] $P < 0.001$, significant difference from the control.

those of the lentinan and curdlan (containing triple-stranded helix).[7,14] Due to the relatively low-molecular weight of the degraded glucan, the intensity of the shift of λ_{max} was much lower than that of lentinan. These results indicated the presence of a low-organized conformation in the native polysaccharide and a relatively high-ordered conformation in the degraded glucan.

Previous studies have shown that a high degree of branching, an adequate size of side chains, and proper conformation in aqueous solution seemed to correlate to the biological activities of β-D-glucans.[14–18] Therefore, the native and degraded glucans were submitted to immunological assays in mice to investigate the correlation between structure and immunological activities (Table 2). As shown in Table 2, it was evident that the degraded glucan had stronger immunological activities than those of the native polysaccharide in view of the lymphocyte proliferation (T and B cells) and the production of antibodies against sheep red blood cells (SRBC) in mice. However, the degraded glucan had no noticeable effect on serum IgG and complement (C-3) levels, and the native glucan showed remarkable effect on enhancing the serum C-3 level in mice.

3. Discussion

Branched β-D-(1 → 3)-linked glucans have been found in several sources, and the funda-

mental structure of the glucan SP from the spores of *G. lucidum* was found to be analogous to that of other β-D-(1→3)-linked glucans having immunomodulating and antitumor activities, such as lentinan, shizophyllan, scleroglucan, etc.[19,20] In structural details, it seemed to differ (the different degree of branching and the length of the side chains) from the others.[19,20] The degraded glucan SP1 had a more linear structure and showed a more ordered conformation in aqueous solution than those of the native glucan SP.

T and B lymphocytes are two important classes of immunologically active cells. The former is mainly responsible for cellular immunity, and the latter is the only cell capable of producing antibodies. Some investigators have reported that polysaccharides isolated from plants or fungi that have immunomodulating properties, including the enhancement of lymphocyte proliferation and antibody production.[21,22] SP and SP-1 were also thought to be such immunoactivating polysaccharides. However, they showed immunostimulating activities in different levels and through different approaches (SP-1 showed a remarkable effect on serum C-3 level). In view of their structural features and conformations in aqueous solution, it was suggested that the degree of substitution on the backbone chain and the length of the side chains might be very important factors in determining the conformation in aqueous solution as well as having an effect on the biological activities of β-D-(1→3)-linked glucans from fungi. This deduction was in agreement with the previous reports.[14,15,18]

4. Experimental

Materials.—Air-dried sporoderm-broken spores of *G. lucidum* (1.0 kg) were supplied by the Green Valley Company (Shanghai, People's Republic of China). They were identified by Professor Xiu-lan Huang, who has deposited them in the Phytochemistry Department of Shanghai Institute of Materia Medica, Chinese Academy of Sciences. 4-[2-[Cyclohexylcarbonimidoyl)amino]ethyl]-4-methylmorpholinium 4-methylbenzenesulfonate (1-cyclohexyl-3-(2-morpholinoethyl) carbodiimide metho-*p*-toluene sulfonate, CMC), trifluoroacetic acid (TFA) and dimethyl sulfoxide (DMSO) were from E. Merck. Concanavalin A (ConA) and lipopolysaccharide (LPS) were from Sigma Chemical Co., and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was from Fluka. Medium RPMI-1640 was purchased from Gibco Laboratories. All RMPI-1640 media were supplemented with HEPES buffer 10 mM, benzylpenicilin 100 IU/L, streptomycin 100 mg/L, L-glutamine 2 mM, 2-mercaptoethanol 50 μM and 10% newborn bovine serum, pH 7.2. All other reagents were of the highest available quality.

Isolation and purification of the polysaccharide.—After the sporoderm-broken spores of *G. lucidum* were defatted by 95% alcohol, the residue was decocted for 4 h with 2 × 20 vols of water. The combined aq extract was deproteinated with trichloroacetic acid.[8] After centrifugation, the supernatant solution was intensively dialyzed against running water for 3 days and then distilled water for 1 day (cut off 3000 Da). The nondialyzate fraction was concentrated under reduced pressure to 1.8 L, and 4 vols of 95% alcohol were added slowly and stirred at 4 °C. Then the mixture was stored overnight at −10 °C. The resulting precipitate, obtained by centrifugation, was dissolved in water (0.5 L) at rt, and further fractionated by precipitation with EtOH (0.5 L). After centrifugation, the remaining supernatant was totally precipitated by the addition of another 3 vols of EtOH at 4 °C. The resulting precipitate was vacuum-dried at 40 °C and gave a yellow power (yield: 1.06%). A portion of the crude polysaccharide fraction (10.5 g) was applied to a DEAE-cellulose column (10 × 50 cm) eluting with water, followed by a linear gradient up to 1 M NaCl. Based on the colorimetric test for total sugar with the phenol–H_2SO_4 method, the fraction eluted with water was further purified on a column of Sephacryl S-200 HR to give a polysaccharide (2.4 g), eluted with 0.2 M NaCl, whose chemical homogeneity was shown by HPSEC.

General procedures.—^1H and ^{13}C NMR spectra were obtained at 400 and 100 MHz, respectively, with a Bruker AM 400 spectrometer equipped with a dual probe in the FT

mode at rt. The ^{13}C NMR chemical shifts were measured using CH_3OH as an internal standard (δ 49.78 relative to the signal of TMS). The DEPT experiments were performed using a polarization-transfer pulse of 135°. Specific rotation was determined in water with a Perklin–Elmer 241M digital polarimeter. IR spectra were carried out on a Perkin–Elmer 591B with KBr or Nujol pellets. HPSEC was performed with a Waters instrument fitted with GPC software, using a Waters 2410 RID as the detector. GLC was carried out on a Shimadzu-9A apparatus equipped with a 5% OV 225/AW-DMC-Chromosorb W (80–100 mesh) column. GLC–MS spectra were obtained with a MD 800 instrument fitted with an HP-1 column. Visible absorption spectra were recorded with an UV-260 Shimadzu spectrophotometer. ESIMS spectra were preformed with a VG Quattro MS/MS spectrometer.

Homogeneity and molecular weight.—These were determined by HPSEC fitted with GPC software on a column of Ultrahydrogel™ 1000, eluted with 0.001 M NaOH. The column was pre-calibrated using standard T-Dextrans (T-500, T-110, T-80, T-70, T-40 and T-9.3). All samples were prepared as 0.1% (w/v) solutions and 20 μL of solution was injected in each run.

Compositional analysis.—The sugar constituent analysis was determined by TLC and GLC as previously described.[9]

Methylation analysis.—The polysaccharide was methylated three times by the Needs and Selvendran method.[11] The pre-methylated product was depolymerized with 90% HCOOH at 100 °C for 6 h and further hydrolyzed with 2 M TFA at 100 °C for 2 h.[7,8] The partially methylated residues were reduced and acetylated by using the method described by Blakeney et al.[9] The resulting products were analyzed by GLC–MS. The GLC temperature program was isothermal at 140 °C for 3 min, followed by a 3 °C/min gradient up to 250 °C. Methylated alditol acetates were identified by their fragment ions in EIMS and by relative retention times on GLC, and the molar ratios were estimated from the peak areas and response factors.[23]

Periodate oxidation and Smith degradation.—The native polysaccharide SP (300 mg) was oxidized with 0.02 M $NaIO_4$ (300 mL) for 7 days at 5 °C in the dark. Consumption of $NaIO_4$ was measured by spectrophotometric method.[24] After 7 days reaction, the consumption of $NaIO_4$ was kept to a constant level. The resulting mixture was neutralized, reduced, and mild hydrolyzed with 0.2 M TFA at 40 °C for 24 h. After dialysis of the hydrolysate, a nondialysate portion (SP-1, yield: 52.3%) and a dialysate portion were obtained. The dialysate portion was further fractionated by Sephadex G-10 chromatography. According to the carbohydrate profile, three oligosaccharide fractions were obtained.

Complex formation with Congo Red.—The complexation with Congo Red was evaluated by the wavelength shift at the maximum visible absorption of Congo Red that was induced by the presence of polysaccharides at various concentrations of alkali according to the procedures of Ogawa et al.[13] Polysaccharides in an NaOH solution (1.0 g/L) were mixed with an equal volume of Congo Red solution (6.1 mM). The concentration of alkali was stepwise increased from 0 to 0.5 M.

Lymphocyte proliferation in mice.—Inbred ICR (female) mice were obtained from Shanghai Experimental Animal Laboratory, Chinese Academy of Sciences (certificate no. 153), body weight 20 ± 2 g. They were divided randomly into three groups: normal saline (control), native polysaccharide group (SP) and degraded polysaccharide group (SP-1). The mice were given samples by intraperitoneal injection (ip) at a dose of 50 mg/kg for 4 days, respectively. On day 5, the mice were sacrificed and their spleens were removed, minced and passed through a stainless mesh (200 mesh) to obtain single-cell suspensions. Following a threefold wash with RPMI 1640, the spleen cells were suspended to a final density of 5.0×10^9 cells/L in a RPMI 1640 medium. Cells (100 μL/well) were seeded into a 96-well plate in the presence of mitogen ConA (5.0 μg/mL) or LPS (25 μg/mL). After incubation for 44 h at 37 °C in a humidified 5% CO_2 incubator, T and B lymphocyte proliferation was assayed by the MTT method.[22,25] The plate was incubated for another 4 h, and the resolver (100 μL/well) was then added. The absorbance was measured by DG-3022 ELISA at 570 nm.

Antibody production and serum IgG and complement (C3) assays in mice.—The effect on antibody production was measured using the quantitative hemolysin spectrophotometric (QHS) assay.[26] The mice were challenged by ip of 0.2 mL of 5% sheep red blood cells (SRBC) on day 0. The samples of SP and SP-1 were injected ip into the mice from days 1 to 4 at a dose of 50 mg/kg, respectively. For the control group, the normal saline was administrated. The mice were killed on day 5 to obtain serum for antibody production assay, IgG and C-3 determinations. Fresh SRBC was washed twice with PBS (pH 7.2) and diluted to 1:20. A suspension of spleen cells (2×10^7 cells/mL) was prepared. Cell suspensions 1.0 mL, SRBC 1.0 mL and pooled serum of guinea-pig 1.0 mL were mixed and incubated at 37 °C for 1.5 h and then centrifuged. Absorbance at 520 nm of the supernatants was measured.

Serum IgG and C-3 levels were determined by the single immunodiffusion method described previously.[26] The diameters of samples in a rabbit anti-mouse serum plate ring were measured.

Acknowledgements

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