Purification, Characterization, and Modification of T Lymphocyte-Stimulating Polysaccharide from Spores of *Ganoderma lucidum*

Xing-Feng BAO, Yun ZHEN, Li RUAN, and Ji-Nian FANG*

Shanghai Institute of Materia Medica, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences; 294 Taiyuan Road, Shanghai 200031, PR China. Received December 10, 2001; accepted February 6, 2002

The hot-water extract of the spores of *Ganoderma lucidum* was shown to have a stimulating effect on concanavalin A-induced mitogenic activity of T lymphocytes. Bioassay-guided separation led to the isolation of a polysaccharide with potent T lymphocyte-stimulating activity by ethanol fractionation, anion-exchange, and size-exclusion chromatography. Based on the composition and methylation analyses, periodate oxidation, Smith degradation, and NMR spectroscopy, the native polysaccharide was shown to be a β-D-(1→3)-glucan with branches of terminal glucosyl residues substituted at C-6 of the glucose residues in the main chain. The branching ratio is approximately 20%. A series of sulfated or carboxymethylated derivatives were prepared and their structural features were elucidated by chemical and spectral analyses. The solution conformation and T lymphocyte proliferation effect of the glucans before and after derivatization were compared and discussed. The data obtained indicate that the introduction of ionic groups would significantly affect the original conformation of the native glucan in aqueous solution and further affect T lymphocyte-stimulating activity. The triple-helical structure of the glucans, the nature of the ionic groups, and the density of negative charge were considered to be closely related to this activity.

Key words *Ganoderma lucidum*; polysaccharide; T lymphocyte-stimulating activity; sulfation; carboxymethylation; solution conformation

Ganoderma lucidum, a well-known Chinese medicinal fungus, has been used to promote health and longevity in East Asian countries. Its medical effects on cancer, hypertension, hepatitis, and hypercholesterolemia have been demonstrated by pharmacological studies in the past two decades.[1—4] This fungus has attracted considerable attention partly because the polysaccharides isolated from the fruit bodies and the mycelium have shown antitumor and hyperglycemic activity.[3,5,6] However, the active components in the spores of *G. lucidum* have rarely been studied owing to the difficulty in collection and sporoderm-breaking of the spores. Recently, with the successful cultivation of *G. lucidum* indoors on a large scale and a breakthrough in sporoderm-breaking technology, much attention has been paid to chemical components of *G. lucidum* spores[7,8] and their versatile biological activities.[9,10] More recently, we have reported an immunomodulating polysaccharide from the spores of *G. lucidum*.[11] In the present study, we deal with the purification and characterization of the T lymphocyte-stimulating polysaccharide, based on the activity-guided principle, from the hot-water extract of the sporoderm-broken spores of *G. lucidum*. A series of sulfated or carboxymethylated derivatives of the polysaccharide were successively prepared and their structures were elucidated by chemical and spectral methods. The solution conformation and T lymphocyte proliferation effect of the polysaccharide and its derivatives are compared and discussed.

Experimental

Materials Spores of *G. lucidum* were collected in Shanxi Province, PR China. It was identified by Dr. Xiu-Lan Huang and stored as a voucher specimen (no. 99064) in the Herbarium of Phytochemistry Department of Shanghai Institute of Materia Medica, Shanghai, PR China. Before use, the sporoderm of the spores was ultrasonographically broken and the broken ratio was estimated about 60—80% by electronic microscopy. T-Dextran in a series of different molecular weights was obtained from Pharmacia. Concanavalin (Con A) was from Sigma and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was from Fluka. Medium RPMI-1640 was purchased from Gibco Laboratories. All other reagents were of the highest quality available and were used without further purification.

Isolation and Purification of the T Lymphocyte-Stimulating Polysaccharide The sporoderm-broken spores of *G. lucidum* (1.0 kg), previously defatted with 95% alcohol, were decocted for 6 h with 10 l of water to half the original volume, and the residue was again decocted with 8 l of water. The combined aqueous extract was deproteinated with trichloroacetic acid, and the resulting aqueous fraction was extensively dialyzed against running water for 3 d and then against distilled water for 1 d (molecular weight cut-off 3000—5000 Da). The retentate was concentrated under reduced pressure to a small volume (1.8 l), and 4 volumes of ethanol were added stepwise with stirring at 4 °C. Then the mixture was stored overnight at −10 °C. The resulting precipitate obtained by centrifugation was dissolved in water (500 ml) and further fractionated by precipitation with EtOH (30, 50, 80%). After centrifugation, each fraction was collected and vacuum-dried at 40 °C. The active fraction was obtained with 30% ethanol precipitation (PSGL-I, yield: 5.7 g).

To purify T lymphocyte-stimulating polysaccharide, sequential column chromatography was performed with PSGL-I. Each fraction with higher T lymphocyte-stimulating activity was repeatedly subfractionated. PSGL-I was applied to a DEAE-cellulose column (Cl^- form, 5.0×50 cm), eluted with H_2O, followed stepwise by 0.1, 0.3, 0.5, and 2.0 M NaCl. No carbohydrate was detected by phenol-sulfuric acid color reaction in the fractions eluted with 0.5 and 2.0 M NaCl. Thus the corresponding fractions, PSGL-I-1 (3.6 g), PSGL-I-2 (0.7 g), and PSGL-I-3 (0.3 g) were pooled, dialyzed, and lyophilized. PSGL-I-1 with the highest activity was further fractionated on a Sephacryl S-300 HR column (2.6×90 cm) eluted with 0.2 M NaCl, resulting in two fractions (PSGL-I-1A and PSGL-I-1B). The more active PSGL-I-1A fraction was purified by rechromatography on the same exclusion column (yield: 1.5 g).

General Analysis The carbohydrate and protein contents were measured by the phenol-sulfuric acid method and the Lowry experiment, respectively. The specific rotation was determined on a Perkin-Elmer 241M digital polarimeter in water at 20±1 °C. IR spectra (KBr or Nujol pellets) were recorded on a Perkin-Elmer 599B FTIR spectrometer. Gas chromatography (GC) was carried out with a Shimadzu GC-14BPF apparatus, equipped with a 5% OV225/AW-DMCS-Chromosorb W (80—100 mesh) column (2.5 m×3 mm), as well as a hydrogen-flame ionization detector. GC-MS was conducted with a Finnigan Model MD-800 combined with GC-MS spectrometry equipped with an HP-1 capillary column. High performance size exclusion chromatography (HPSEC) was performed with a Waters instrument fitted with GPC software, using a Waters 2410 as detector. The ^1H- and ^{13}C-

* To whom correspondence should be addressed. e-mail: jnfang@mail.shcnc.ac.cn

NMR spectra were obtained at 400 and 100 MHz in D_2O, respectively, with a Bruker AM400 spectrometer with a dual probe in the FT mode at room temperature. The distortionless enhancement by polarization transfer (DEPT) experiments were done using a polarization transfer pulse of 135°. UV and visible absorption spectra were recorded with a Shimadzu UV-260 spectrophotometer.

Homogeneity and Molecular Weight Measurements were made with HPSEC on a linked column of Ultrahydrogel 500 and Ultrahydrogel 2000, using 0.003 M NaOAc as the mobile phase at a flow rate of 0.5 ml/min. The column was 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 analyzed in each run.

Compositional and Methylation Analyses The sugar composition was analyzed by GC after conversion of the hydrolysate into alditol acetates as described by Blakeney *et al*.[12] The polysaccharide samples were methylated three times by the method of Needs and Selvandran,[13] and the resulting per-methylated product was hydrolyzed, reduced, acetylated, and analyzed by GC-MS as described previously.[14] The partially methylated alditol acetates were identified by their fragment ions in electron impact (EI)-MS and by relative retention times in GC, and the molar ratios were estimated from the peak areas and response factors.[15]

Periodate Oxidation and Smith Degradation The polysaccharide (50 mg) was dissolved in 0.02 M $NaIO_4$ (50 ml) and stored in the dark at 5 °C. Absorption at 224 nm was detected every day. The oxidation was completed 5 d later, and ethylene (0.5 ml) was added to the solution. Consumption of $NaIO_4$ was measured by a spectrophotometric method[16] and HCOOH production by titration with 0.01 M sodium hydroxide. The reaction mixture was reduced by $NaBH_4$, neutralized by 0.1 M HOAc, dialyzed against distilled water, and the retentate was then lyophilized to give a product (PSGL-I-1A-O1, yield: 41 mg). The molar ratio of glycerol to glucose residues in the resulting product was analyzed by GC as alditol acetates.[17] The GC temperature program was isothermal at 190 °C for 5 min and then a gradient up to 210 °C. PSGL-I-1A-O1 was further hydrolyzed by 0.1 M CF_3COOH at 40 °C for 24 h.[18] After hydrolysis, a water-insoluble product (PSGL-I-1A-O2, yield: 26 mg) was obtained by centrifugation and vacuum-dried. It was then subjected to methylation analysis.

Sulfation of the Polysaccharide The sulfating reagent was prepared using piperidine and chlorosulfonic acid as described by Nagasawa and Yoshidome.[19] The polysaccharide (100 mg) was suspended in dry Me_2SO (10 ml) with stirring for 30 min at room temperature and then the temperature was increased in gradients up to 85 °C. Subsequently, a specific amount of piperidine *N*-sulfonic acid was added and the reaction time was varied to obtain the sulfated derivatives with different degrees of substitution (Table 1). After the reaction was completed, the mixture was cooled and neutralized with saturated $NaHCO_3$. The product was isolated by extensive dialysis against saturated $NaHCO_3$ for 1 d and against distilled water for another 3 d, and then the retentate was lyophilized to give the naturally sulfated sample. The degree of substitution was estimated by the method of Silvestri *et al*.[20]

Carboxymethylation of the Polysaccharide The polysaccharide was carboxymethylated according to the procedures of Adachi *et al*.[21] Briefly, the polysaccharide (100 mg) was suspended in 2-propanol (5 ml) with stirring for 15 min at room temperature. Then 0.5 ml of 30% sodium hydroxide was added slowly within 5 min and vigorous stirring continued for 90 min. Subsequently, chloroacetic acid (0.24 g) was added to the mixture and the reaction temperature was varied to obtained the desired products (Table 2). After the reaction was stopped 3 h later, the mixture was cooled, neutralized, dialyzed against distilled water, and lyophilized to give the carboxymethylated product. The degree of substitution was measured by classical titration and by ^{1}H-NMR analysis.

Acid Hydrolysis of Carboxymethylated Polysaccharides Hydrolysis was achieved by heating the carboxymethylated derivatives (each 30 mg) in 2.4 M perchloric acid (10 ml) at 65 °C for one week.[22] After hydrolysis, the resulting solution was cooled in an ice-bath, neutralized with potassium hydroxide, and the precipitate of potassium perchlorate was removed by centrifugation. The water was then removed with a rotatory evaporator and the solid residue was vacuum-dried. Each dried sample was dissolved with 0.5 ml D_2O plus 0.1 ml D_2SO_4 and then subjected to NMR measurement. The application of this solvent was used to shift the residual HOD signal upfield (>6.5 ppm).

Complex Conformation with Congo Red The change in absorption of Congo Red in the presence of the polysaccharide or its derivatives was measured by the procedures of Ogawa *et al*.[23] Each sample solution (2 mg/ml of sodium hydroxide) and 12.2 μM of Congo Red were mixed in equal volumes, and the absorption maximun (λ_{max}) was measured at room temperature.

T Lymphocyte-Stimulating Activity Assay Inbred ICR female mice were obtained from the Shanghai Experimental Animal Laboratory (certificate no. 153), Chinese Academy of Sciences, Shanghai, PR China. The effect of polysaccharide samples on T lymphocyte proliferation *in vitro* and in mice was measured by the MTT method as described elsewhere.[14,24] All data are expressed as mean±S.D., and the significance of difference between the means was evaluated by Student's *t*-test. The cytotoxicity of all samples to T lymphocytes at concentrations of 1—100 μg/ml was also evaluated by the MTT method in the absence of Con A *in vitro*.[24]

Table 1. Sulfation of PSGL-I-1A with Piperidine *N*-Sulfonic Acid[a]

Derivative[b]	[DNS]/[PSGL-I-1A][c]	Time (min)	Yield (mg)	S (%)	DS	MW ($\times10^{-5}$)
P-SA-1	2.5	45	112	8.7	0.6	2.0
P-SA-2	5.0	45	168	12.9	1.1	1.3
P-SA-3	5.0	75	160	14.4	1.4	0.5
P-SA-4	10.0	60	113	16.6	1.9	0.4

a) The reaction temperature was 85 °C. *b*) From the parent glucan PSGL-I-1A 100 mg. *c*) The initial ratio of piperidine *N*-sulfonic acid to PSGL-I-1A monomer units.

Results and Discussion

Purification of T Lymphocyte-Stimulating Polysaccharide The sporoderm-broken spores of *G. lucidum* were defatted with 95% alcohol, and then the insoluble portion was solubilized with hot water. The hot-water extract was primarily fractionated by ethanol precipitation at different concentrations and gave three fractions, PSGL-I (30% EtOH), PSGL-II (50%), and PSGL-III (80%), among which the highest T lymphocyte-stimulating activity was observed in the PSGL-I fraction. PSGL-I was further fractionated on a DEAE-cellulose column with water and different concentrations of stepwise NaCl solution elution (0.1, 0.3, 0.5, and 2.0 M NaCl), leading to the isolation of the three carbohydrate-containing subfractions PSGL-I-1, PSGL-I-2, and PSGL-I-3. Of those, fraction PSGL-I-1 showed the relatively high activity. Therefore PSGL-I-1 was repeatedly subjected

Table 2. Carboxymethylation of PSGL-I-1A with Chloroacetic Acid[a]

Derivative[b]	Temp. (°C)	Time (h)	Yield (mg)	MW ($\times10^{-6}$)	DS		DS_i[f]			
					DS_{tit}[d]	DS_{NMR}[e]	DS_2	DS_3	DS_4	DS_6
P-CM-1	30	3	113	0.9	0.32	0.35	0.04	n.r.[g]	0.10	0.21
P-CM-2	50—30[c]	1—2[c]	122	1.4	0.69	0.72	0.10	n.r.	0.25	0.37
P-CM-3	50	3	137	1.6	1.07	1.11	0.22	n.r.	0.40	0.49
P-CM-4	70	3	146	2.5	1.35	1.43	0.29	n.r.	0.57	0.57

a) The initial ratios of chloroacetic acid and NaOH to PDGL-I-1A monomer units were fixed at 4 and 6, respectively. *b*) From the parent glucan PSGL-I-1A 100 mg. *c*) The reaction continued at 50 °C for 1 h and then 30 °C for another 2 h. *d*) Values obtained from classical titration. *e*) Values obtained from ^{1}H-NMR measurement. *f*) DS_i=partial DS at C-i position. *g*) n.r., not resolved.

to size-exclusion column chromatography on Sephacryl S-300 to give two polysaccharides, PSGL-I-1A and PSGL-I-1B. The relatively low-molecular sized polysaccharide PSGL-I-1A displayed greater T lymphocyte-stimulating activity. The results of T lymphocyte proliferation tests for each fraction are summarized in Table 3. As shown in Fig. 1 and Table 3, the polysaccharide PSGL-I-1A showed a significantly enhancing effect on Con A-induced T lymphocyte proliferation both *in vitro* and in mice at the experimental dose range. This indicates that PSGL-I-1A is a potent T lymphocyte stimulator. The HPSEC profile showed a single and symmetrically sharp peak (data not shown), suggesting that PSGL-1A is a homogeneous polysaccharide with an average molecular weight of 7.18×10^5 Da. A negative response to the Lowry experiment indicated that the polysaccharide contained no protein.

Structural Characterization of PSGL-I-1A Sugar compositional analysis showed that PSGL-I-1A was only composed of D-glucose. The low negative specific rotation, $[\alpha]$ −23.4° (c=0.86, H_2O), and the characteristic absorption at 893 cm^{-1} in the IR spectrum (Fig. 2A) were indicative of the β-D-glucosidic linkages.

Methylation analysis of PSGL-I-1A revealed the presence of three components as partially methylated alditol acetates, and the identification of di-*O*-methyl glucosyl residues indicated that the native glucan was a branched polymer. Three products were identified as derivatives of 2,3,4,6-tetra-, 2,4,6-tri-, and 2,4-di-*O*-methyl-glucose in a close molar ratio of 1 : 4 : 1. This result suggests that the intact glucan contains a backbone composed of (1→3) linkages with branches of side chains through position 6.

The ^{13}C-NMR spectrum of PSGL-I-1A (Fig. 3A) was very similar to that of fungal β-D-(1→3)-glucans.[26,27] The branching points at C-6 were shown by the signal of an *O*-substituted carbon atom at δ 70.5 and unsubstituted C-6 at δ 62.9. The broad peak at δ 86.4 was ascribed to the presence of linear β-D-(1→3)- and branched β-D-(1→3,6)-linked glucopyranosyl residues. The signals at δ 76.8 and 71.7 could be attributed to unsubstituted C-3 and C-4 of glucosyl residues in branches. Other peaks in the spectrum at δ 77.7, 75.4, and 70.3 were assigned to unsubstituted C-5, C-2 and C-4 of glucosyl residues in the backbone chain, respectively. All those resonances indicated a branched β-D-(1→3)-glucan structure of the native polysaccharide. This result was in accordance with the methylation analysis.

PSGL-I-1A consumed 0.35 mol of periodate and liberated 0.18 mol of formic acid per sugar residue. The oxidized product was reduced by $NaBH_4$, and the resulting polyalcohol (PSGL-I-1A-O1) on complete hydrolysis with acid gave glycerol and glucose in the molar ratio of 1.0 : 4.2. The glycerol was released from the oxidized terminal glucosyl residues, and the survival of glucose was indicative of (1→3) linkages in the native glucan. Mild hydrolysis of the polyalcohol yielded a product (PSGL-I-1A-O2) that was insoluble in water. Methylation analysis indicated that it was almost totally composed of (1→3)-linked glucosyl residues (>98%), together with some components in trace amounts (<2%). This result supported the β-D-(1→3)-linked backbone of the native glucan.

From the foregoing results, PSGL-I-1A appears to contain a backbone of (1→3)-linked glucopyranosyl residues with one (1→6)-linked terminal glucosyl side chain for every six glucosyl residues. The putative structure is shown in Chart 1.

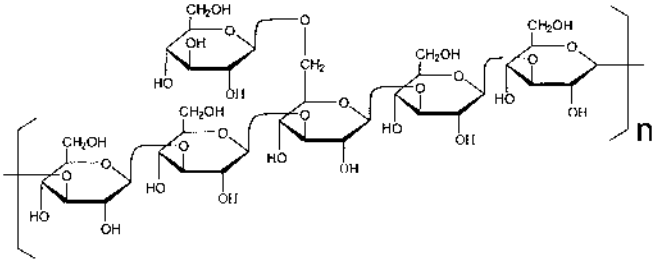

Chart 1. Putative Structure of PSGL-I-1A

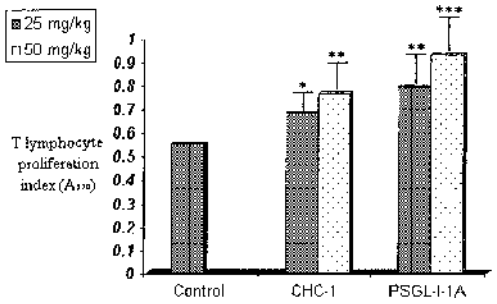

Fig. 1. Effect of PSGL-I-1A on T Lymphocyte Proliferation in Mice

The mice were given samples by intraperitoneal injection of a dose of 25 or 50 mg/kg for 4 successive days. Mitogenic polysaccharide CHC-1 obtained from *Cuscuta chinensis* was used as a positive control.[25] * p<0.05, ** p<0.01, *** p<0.001, significantly different from the control group.

Chemical Characterization of Sulfated Derivatives of

Table 3. Effects of Fractions and Subfractions from the Hot-Water Extract of *G. lucidum* Spores on the Proliferation of T Lymphocytes Induced by Con A in ICR Mouse Splenocytes *in Vitro*

Sample	Control	Concentration (μg/ml)		
		1	10	100
PSGL-I	0.66±0.05	0.68±0.05	0.80±0.03*	0.87±0.01**
PSGL-II	0.71±0.02	0.73±0.02	0.67±0.01	0.61±0.02*
PSGL-III	0.85±0.02	0.87±0.04	0.94±0.01*	0.97±0.02*
PSGL-I-1	0.85±0.02	1.01±0.06	1.10±0.02**	1.16±0.03**
PSGL-I-2	0.66±0.01	0.68±0.03	0.73±0.03	0.79±0.02*
PSGL-I-3	0.66±0.01	0.68±0.01	0.68±0.01	0.70±0.02
PSGL-I-1A	0.64±0.03	0.76±0.02*	0.87±0.01**	0.95±0.01***
PSGL-I-1B	0.64±0.03	0.71±0.03	0.85±0.02**	0.89±0.01**

Results are expressed as mean±S.D. based on three independent experiments. * p<0.05, ** p<0.01, *** p<0.001, significantly different from the control.

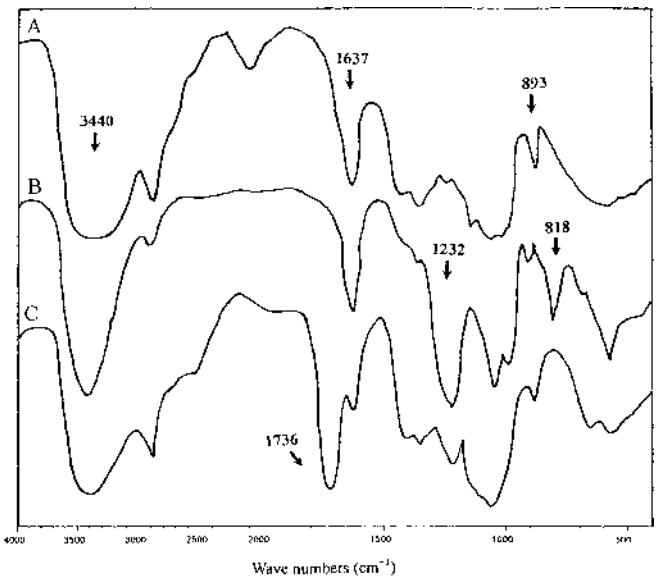

Fig. 2. Comparison of IR Spectra of (A) the Parent Glucan PSGL-I-1A, (B) Sulfated PSGL-I-1A (DS=1.1), and (C) Carboxymethylated PSGL-I-1A (DS=1.11)

PSGL-I-1A A series of sulfated samples with different degrees of substitution (DS) were obtained by varying the molar ratio of piperidine *N*-sulfonic acid to PSGL-I-1A monomers and reaction time (Table 1), according to the method of Yoshida *et al*.[28] The DS that designated the average number of sulfate (SA) groups in each glucosyl residue was calculated from the data obtained by sulfur content analysis. As shown in Table 1, the apparent molecular weight of the derivatives decreased sharply with the increasing DS values in the range from 2.0×10^5 to 0.4×10^5, while their water solubility was considerably increased in comparison with that of the native glucan. It was worthy noting that, since the tested samples had different electrical charges from the standards (neutral dextrans), the apparent values should be considered as relative. The IR spectrum of sulfated samples (DS=1.1) is shown in Fig. 2B. In comparison with that of the parent glucan, the OH stretching vibration bands of *ca.* 3440 cm^{-1} for the sulfated derivatives were narrowed, and two new absorption peaks at 818 cm^{-1} and 1232 cm^{-1} appeared due to the presence of the bonds of C–S–O and S=O, respectively, indicating that the sulfation reaction had occurred. The intensity of the peaks at *ca.* 820 cm^{-1} and *ca.* 1230 cm^{-1} increased in the order P-SA-1<P-SA-2<P-SA-3<P-SA-4, which was in accordance with the results of sulfur content analysis for the sulfated derivative (Table 1).

The ^{13}C-NMR spectrum of sulfated PSGL-I-1A (DS=1.1) is shown in Fig. 3B. By comparison with the spectrum of PSGL-I-1A, a series of new signals appeared resulting from the sulfation of the hydroxyl group at positions C-6, C-2, and C-4. It is known that the modification of the OH group with sulfate in glucosyl compounds entails both a strong downfield shift of the carbon bearing the OH group itself (α-effect) and an upfield shift of the adjacent carbon (β-effect) with respect to carbons with unsubstituted OH groups. Thus, in combination with published data on sulfated polysaccharides,[29,30] the above peaks were assigned as follows: at δ 69.8 to the carbons C6(6) (bearing a sulfate group) and thus reflecting an α-effect; at δ 76.1 to the carbons C5(6) adjacent

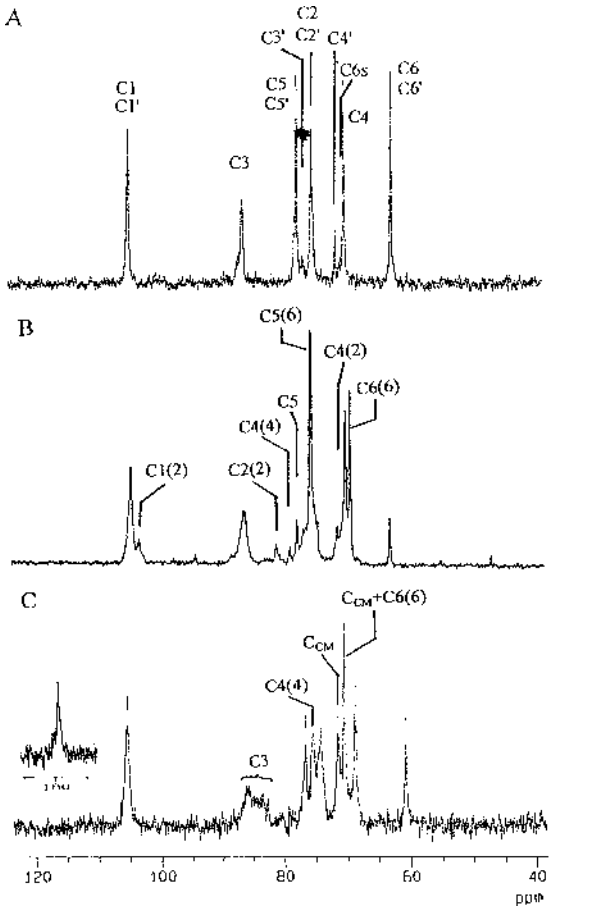

Fig. 3. Comparison of ^{13}C-NMR Spectra of (A) the Parent Glucan PSGL-I-1A, (B) Sulfated PSGL-I-1A (DS=1.1), and (C) Carboxymethylated PSGL-I-1A (DS=1.11)

C6s refers to the C-6 of the branching point unit and C_{CM} refers to the methylenic carbon of the carboxymethyl group.

to substituted C-6 carbons (reflecting a β-effect); at δ 81.6 to the C2(2) carbons (α-effect); at δ 71.9 to the C4(2) carbons (γ-effect); at δ 79.3 to the C4(4) carbons (α-effect); at δ 78.1 to the C2(4) carbons (γ-effect); and at δ 103.7 to the C1(2) carbons (β-effect). Theoretically, position 3 of the terminal glucosyl side chains could also be substituted, although the signal of O-substituted C-3 was not distinct, mainly because of its low content and steric hindrance. Also, it was found that the signal intensity of the carbon C-1 decreased with increasing degree of conversion, while that of the carbons C1(2) increased. Thus the NMR data indicate that the sulfation of PSGL-I-1A was not very selective, but the peak of C6(6) was much more intense than those of C2(2) and C4(4), suggesting that substitution with sulfate at C-6 was the dominant reaction compared with other possible positions in a glucosyl residue.

Chemical Characterization of Carboxymethylated Derivatives of PSGL-I-1A Four carboxymethylated samples with different DS ranging from 0.35 to 1.43 were obtained by varying the reaction temperature with fixed ratios of the reactants (Table 2). As seen in Table 2, the apparent molecular weight of the derivatives increased with increasing DS, which was different from that of the sulfated samples, indicating that the carboxymethylation did not induce the marked decrease in carbohydrate polymer.

The IR spectrum (Fig. 2C) of carboxymethylated PSGL-I-1A (DS=1.11) shows a characteristic peak at 1736 cm^{-1}, suggesting that the carboxymethyl group has been incorporated into the parent glucan. As shown in Fig. 3C, the signal at δ 179.3 in the ^{13}C-NMR spectrum confirms the presence of a carboxyl group, and this occurs together with the appearance of three new signals in comparison with the spectrum of PSGL-I-1A (Fig. 3A). According to the substitution effects (α-, β-, and γ-effects) described above, the signal at δ 76.7 was assigned to C4(4) carbons, the peak at δ 72.9 was ascribed to the methylenic carbon of the carboxymethyl group, and the signal at δ 71.9 was formed by the overlap of partial methylene of carboxymethyl groups and C6(6) carbons. Moreover, the broad and multiple signals of C3 reflect the substitution with the carboxymethyl group at adjacent positions 2 and 4 (β-effect), and the signal for C2(2) was not very distinct, suggesting a relatively low DS value at this position. Thus it could be assumed that positions 4 and 6 within a glucosyl unit were the major reaction sites for the carboxymethylation of PSGL-I-1A.

To investigate further the substitution mode of every free hydroxyl group in a glucosyl residue, the carboxymethylated derivatives were each completely hydrolyzed with perchloric acid, and the residue of each was dissolved in acidic D_2O and then measured by NMR analysis. As seen in Fig. 4, resonances from methylene protons of carboxymethyl substitution are completely resolved from those of other protons. They appear as a set of peaks in the region of δ 4.2—4.6 and correspond to the four possible substitution sites, namely C-2, C-4, C-6, and C-3 (side chain). Thus the DS value can be simply calculated from the ratio of two spectral areas, A/B, where A is one half of the peak areas corresponding to the methylene protons of carboxymethyl substituents in the 4.2—4.6 ppm region and B is the peak areas of all anomeric protons in the 4.6—5.5 ppm region. The data on DS measurements of the different derivatives of PSGL-I-1A are listed in Table 2. In comparison with the DS values measured by titration, generally good agreement was obtained, although the NMR results tended to be slightly higher.

In Fig. 4B, the signals at δ 5.21 and 5.40 were assigned to H1 of α anomers while those at δ 4.65 and 4.72 were attributed to H1 of β anomers. Each signal contains two groups of peaks, which correspond to the different residues resulting from the side chain or backbone of the carboxymethylated glucans. The peaks at δ 4.72 and 5.40 in the ^{1}H-NMR spectrum arose from H1 of residues in which O-2 was carboxymethylated. Peaks arising from anomeric protons of glucosyl units substituted or unsubstituted at O-2 were designated as **s** and **u**, respectively, in Figs. 4A and B. Thus the individual DS_i value at C-2 may be estimated separately from the integration of anomeric protons of residues substituted at C-2. For example, as demonstrated in Fig. 4B, the ratio between downshifted and all anomeric proton resonance is 19.8%. This means that about 20% of hydroxyl groups at C-2 are substituted. Moreover, the carboxymethyl methylene signals corresponding to the substitution at C-6 are also differentiated, which allows the determination of DS_6 directly by integration. However, the differences in chemical shifts of methylene proton peaks of substitution at C-2, C-4, and C-3 (not resolved) are too small to differentiate from each other. As described above, owing to the low content and steric hindrance of C-3 in glucosyl side chains, the substitution at C-3 might be omitted. Therefore the DS_4 value could be calculated by the formulation $DS-(DS_2+DS_6)$. Table 2 shows that the range of DS is between 0.35 and 1.43, and the partial degree of substitution varies almost linearly with DS. The distribution of the carboxymethyl subsituents decreases in the order C-6>C-4>C-2≫C-3, and positions 4 and 6 account for the majority of the reaction sites in a glucosyl residue. This result confirms the speculations based on ^{13}C-NMR measurements.

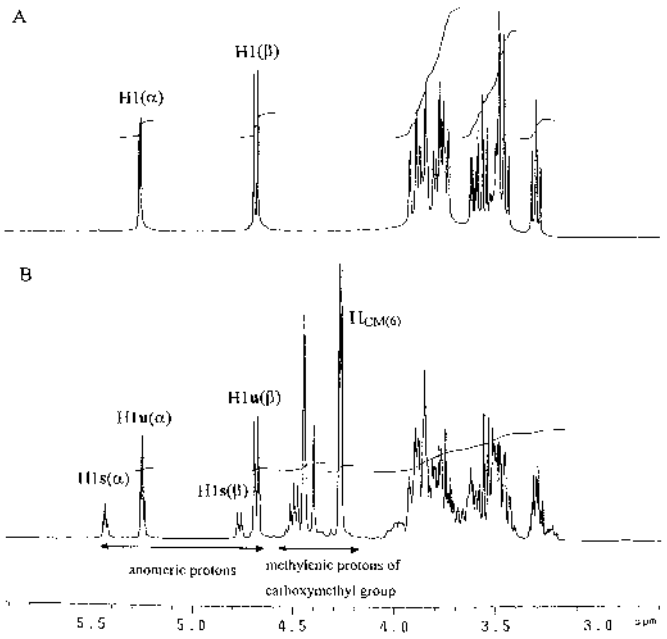

Fig. 4. ^{1}H-NMR Spectra ($D_2O+D_2SO_4$) of (A) Acid-Hydrolyzed PSGL-I-1A and (B) Degraded Carboxymethylated PSGL-I-1A with DS of 1.11

Resonances arising from the anomeric protons of the α and β reducing ends and partial carboxymethyl methylene are indicated. **s** and **u** refer to substituted and unsubstituted units at C-2. $H_{CM(6)}$ is designated as the methylenic protons of the carboxymethyl group substituting at C-6 of a glucosyl residue.

Solution Conformation Investigation of PSGL-I-1A and Its Derivatives The formation of complexes of glucans with Congo Red and the resulting shift of the maximum absorption wavelength (λ_{max}) is a rapid method for detecting changes in helical structures in aqueous solution.[23] Generally, if the wavelength of maximum absorption of the complex with Congo Red shifts to above about 505 nm in 0.1 M sodium hydroxide, it could be considered that the tested polysaccharide contains a triple-helical structure in aqueous solution.[23,31,32] Therefore the maximum absorption of each complex at this alkaline concentration was measured (Table 4). It was clear that the native glucan PSGL-I-1A had a triple-helical conformation, while all other derivatives with carboxymethyl or sulfate group, except P-CM-1, lost the original structure in aqueous solution. The lost of the triple helix indicated that the original intermolecular and intramolecular hydrogen bonds that were responsible for the helix had been partly broken. It is reasonable to consider that a substituted hydroxyl group could no longer form such a cross-linking hydrogen bond.

From Table 4, for carboxymethylation, the decomposition of the ordered structure occurred at DS values between 0.35

and 0.72. Therefore it was assumed that the carboxymethylated derivatives of PSGL-I-1A could basically retain their original structure if the DS value was lower than 0.35, that was, the parent glucan might retain its conformation during carboxymethylation in milder alkaline 2-propanol conditions. However, the helix would be broken under harsh reaction conditions. All sulfated glucans in these experiments lost the ordered conformation because their DS values were too high ($\geq$0.6), which caused the decomposition of the original hydrogen bonds. The increasing charge exclusion enabled the polymer to adopt a stiffer structure in aqueous solutions.[26)]

T Lymphocyte-Stimulating Assay of PSGL-I-1A and Its Derivatives The effects of the parent glucan and its derivatives described above on the mitogenicity of Con A-induced T lymphocytes were tested *in vitro* (Table 5). It was obvious that the native glucan had potent and dose-dependent enhancement of T lymphocyte proliferation at concentrations of 1—100 μg/ml, which agreed with the results of *in vivo* study (Fig. 1). However, this effect sharply decreased (or disappeared) with the introduction of carboxymethyl or sulfate groups. Interestingly, the carboxymethylated PSGL-I-1A with the lowest DS of 0.35 basically preserved the stimulating effect of the native glucan, while the carboxymethylated derivatives with higher DS value ($>$1.0) began to exhibit T lymphocyte-inhibitory activity. All sulfated samples had no noticeable effect on T lymphocyte proliferation. Also, there was no observed toxicity of any of the samples as measured by cell viability at the experimental concentration range.

More intriguingly, the effects of PSGL-I-1A and its derivatives on T lymphocyte proliferation appeared to correlate with the conformation change described above. Samples with a triple-helical structure were found to significantly enhance T lymphocyte proliferation, while other derivatives without the helical conformation lost this stimulatory effect. Thus it was assumed that the triple-helical structure of the glucan was closely related to its T lymphocyte-stimulating activity. With increasing DS values ($>$1.0) of carboxymethyl groups, an opposite action appeared, while no marked effect of sulfated samples was observed, suggesting that the nature of the ionic group and the density of the negative charges were also responsible for the T lymphocyte-stimulation. The reason why sulfated β-D-(1$\rightarrow$3)-glucans did not show any noticeable effect remains to be elucidated. However, if there is a certain structural unit (including conformation and charge) that is specifically recognized by T lymphocytes, it can be speculated that the derivatives could no longer interact with T lymphocytes.

Table 4. The Wavelength Shift of the Maximum Absorption of Complexes with Congo Red in 0.1 mol/l Sodium Hydroxide

Sample	λ_{max} (nm)
Congo Red	488.0
PSGL-I-1A	515.0
P-CM-1	512.6
P-CM-2	491.4
P-CM-3	488.8
P-CM-4	484.0
P-SA-1	494.4
P-SA-2	490.2
P-SA-3	490.8
P-SA-4	490.6

Conclusions

Using the activity-guided separation method, a polysaccharide with a potent T lymphocyte-stimulating effect has been isolated and identified as a branched β-D-(1$\rightarrow$3)-glucan from the hot-water extract of the spores of *G. lucidum*. The fundamental structure of PSGL-I-1A was found to be analogous to that of β-D-(1$\rightarrow$3)-glucans that were reported to have antitumor activity, such as lentinan, schizophyllan, scleroglucan, *etc*.[27,33,34)] In structural details, it appeared to have a relatively low degree of branching (terminal glucosyl side chains) in comparison with the others. Recently, we have reported the structure and immunomodulating activity of two polysaccharides, called SP and PGL, from *G. lucidum* spores. The polysaccharide SP, which was obtained from the fraction of PSGL-III, was a highly branched β-D-(1$\rightarrow$3)-glucan with 1,3-linked oligosaccharide chains. It took a low-organized conformation in aqueous solution and showed less mitogenicity of T lymphocyte in comparison with those of PGSL-I-1A.[14)] The other polysaccharide PGL purified from the PSGL-II fraction had a different backbone of β-D-(1$\rightarrow$6)-linkages branched with 1,3-, 1,4- and terminal glucosyl chains of different size, and unexpectedly, it suppressed T lymphocyte proliferation.[11)] It was suggested that the linkage modes of the backbone of glucans could be a vital factor influencing their immunological activity.

We prepared sulfated and carboxymethylated derivatives of PSGL-I-1A, and the chemical characterization of the modified derivatives indicated that position 6 was the dominant reaction site for sulfation, while the substitution with carboxymethyl group mainly occurred at C-4 and C-6. In both

Table 5. Effect of PSGL-I-1A and Its Derivatives on the Proliferation of T Lymphocytes Induced by Con A in ICR Mouse Splenocytes *in Vitro*

Sample	Control	Concentration (μg/ml)		
		1	10	100
PSGL-I-1A	0.64±0.03	0.76±0.02*	0.87±0.01**	0.95±0.01***
P-CM-1	0.56±0.01	0.69±0.03*	0.75±0.01**	0.84±0.03**
P-CM-2	0.56±0.01	0.60±0.01	0.62±0.04	0.68±0.05
P-CM-3	0.74±0.02	0.70±0.06	0.67±0.04	0.61±0.03*
P-CM-4	0.74±0.02	0.67±0.04	0.60±0.03*	0.57±0.06*
P-SA-1	0.60±0.01	0.60±0.02	0.62±0.08	0.65±0.02
P-SA-2	0.60±0.01	0.64±0.04	0.66±0.07	0.68±0.05
P-SA-3	0.65±0.05	0.58±0.04	0.64±0.05	0.68±0.03
P-SA-4	0.65±0.05	0.59±0.03	0.62±0.05	0.62±0.02

Results are expressed as mean±S.D. based on three independent experiments. * $p<0.05$, ** $p<0.01$, *** $P<0.001$, significantly different from the control.

derivatives, a relatively low DS value at C-2 was observed, mainly because 2-OH was involved in the intermolecular hydrogen bonds and steric hindrance (inside the helix). The relative reactivity of each hydroxyl group in a glucosyl residue observed in the present study fits the results obtained for sulfated and carboxymethylated curdlan.[29,35] The introduction of anion groups into PSGL-I-1A would induce the disordered structure and further affect the T lymphocyte-stimulating activity. For the immunomodulating activity of β-D-(1→3)-glucans, Demleitner *et al.* reported that it is exclusively the β-D-(1→3)-glucosidic linkages that is essential structural feature.[36] Kulicke *et al.* also stated that the helical structures are not essential and not even advantageous for immunological activity.[37] However, our results indicate that the triple-helical structure of β-D-1,3-linked glucans is favorable for T lymphocyte proliferation effect, and the incorporated carboxymethyl or sulfate groups are not advantageous for this activity. In particular, high DS with carboxymethylation results in the opposite effect. Further understanding of the suppressive effect of highly substituted derivatives with carboxymethylation on T lymphocyte proliferation is needed. In current, the research on other immunological parameters of the native glucan and its derivatives is in progress.

Acknowledgments This work was supported by a grant from the Shanghai Committee of Sciences & Technology and partly supported by the Shanghai Institutes for Biological Sciences. The authors are grateful to Professor Xiaoyu Li for her help in immunopharmacological assays and to Shanghai Green Valley Company (Shanghai, PR China) for providing the dried crude materials.

References

1) Franz G., *Planta Med.*, **55**, 493—497 (1989).
2) Furusawa E., Chou S. C., Furusawa S., Hirazami A., Dang Y., *Phytother. Res.*, **6**, 300—304 (1992).
3) Wang S. Y., Hsu M. L., Hsu H. H., Tzeng C. H., Lee S. S., Shiao M. S., Ho C. K., *Int. J. Cancer*, **70**, 699—705 (1997).
4) Kim D. H., Shim S. B., Kim N. J., Jang I. S., *Biol. Pharmacol. Bull.*, **22**, 162—164 (1999).
5) Sone Y., Okuda R., Wada N., *Agri. Biol. Chem.*, **49**, 2647—2653 (1985).
6) Hikino H., Konno C., Mirin Y., Hayashi T., *Planta Med.*, **51**, 339—340 (1985).
7) Min B. S., Nakamura N., Miyashiro H., Bae K. W., Hattori M., *Chem. Pharm. Bull.*, **46**, 1607—1612 (1998).
8) Min B. S., Gao J. J., Nakamura N., Hattori M., *Chem. Pharm. Bull.*, **48**, 1026—1033 (2000).
9) Zhang L. H., Xiao P. G., *Phytother. Res.*, **7**, 217—226 (1993).
10) Zhang L. H., Wang H. X., Wang L. W., Xiao P. G., *Chin. J. Immunol.*, **10**, 169—172 (1994).
11) Bao X. F., Fang J. N., Li X. Y., *Biosci. Biotech. Biochem.*, **65**, 2384—2391 (2001).
12) Blakeney A. B., Harris P. J., Henry R. J., Stone B. A., *Carbohydr. Res.*, **113**, 291—299 (1983).
13) Needs P. W., Selvandran R. R., *Carbohydr. Res.*, **245**, 1—10 (1993).
14) Bao X. F., Liu C. P., Fang J. N., Li X. Y., *Carbohydr. Res.*, **332**, 64—71 (2001).
15) York W. S., Darvill A. G., McNeil M., Stevenson T. T., Albersheim P., *Methods Enzymol.*, **118**, 3—40 (1986).
16) Dixon J. S., Lipkin D., *Anal. Chem.*, **26**, 1092—1093 (1954).
17) Yamada H., *Carbohydr. Res.*, **125**, 107—115 (1984).
18) Bao X. F., Dong Q., Fang J. N., *Acta Biochim. Biophys. Sin.*, **32**, 557—561 (2000).
19) Nagasawa K., Yoshidome H., *Chem. Pharm. Bull.*, **17**, 1316—1323 (1969).
20) Silvestri L. J., Hurst R. E., Simpson L., Settine J. M., *Anal. Biochem.*, **123**, 303—309 (1982).
21) Adachi Y., Ohno N., Ohsawa M., Sato K., Oikawa S., Yadomae T., *Chem. Pharm. Bull.*, **37**, 1838—1843 (1989).
22) Glinel K., Sauvage J. P., Oulyadi H., Huguet J., *Carbohydr. Res.*, **328**, 343—354 (2000).
23) Ogawa K., Tsurugi J., Watanabe T., *Chem. Lett.*, **1972**, 689—692 (1972).
24) Li X. Y., Wang J. F., Zhu P. P., Liu L., Ge J. B., Yang S. X., *Acta Pharmacol. Sin.*, **11**, 542—545 (1990).
25) Wang Z., Fang J. N., Ge D. L., Li X. Y., *Acta Pharmacol. Sin.*, **21**, 1136—1140 (2000).
26) Corinne C., Bruneteau M., *Carbohydr. Res.*, **313**, 259—263 (1998).
27) Yoshioka Y., Tabeta R., Saito H., Uehara N., Fukuoka F., *Carbohydr. Res.*, **140**, 93—100 (1985).
28) Yoshida T., Yasuda Y., Mimura T., Kaneko Y., Nakashima H., Yamamoto N., Uryu T., *Carbohydr. Res.*, **276**, 425—436 (1995).
29) Zhang L. N., Zhang M., Zhou Q., Chen J. H., Zeng F. B., *Biosci. Biotech. Biochem.*, **64**, 2172—2178 (2000).
30) Yoshida T., Hatanaka K., Uryu T., Kaneko Y., Suzuki E., Miyano H., Mimura T., Yoshida O., Yamamoto N., *Macromolecules*, **23**, 3717—3722 (1990).
31) Hara C., Kiho T., Tanaka Y., Ukai S., *Carbohydr. Res.*, **110**, 77—87 (1982).
32) Gandon C., Bruneteau M., *Carbohydr. Res.*, **313**, 259—263 (1998).
33) Sasaki T., Takasuka N., *Carbohydr. Res.*, **47**, 99—104 (1976).
34) Rinaudo M., Vincendon M., *Carbohydr. Res.*, *Carbohydr. Polym.*, **2**, 135—144 (1982).
35) Ukai S., Yoshida I., Honda A., Nagai K., Kiho T., *Carbohydr. Res.*, **224**, 201—208 (1992).
36) Demleitner S., Kraus J., Franz G., *Carbohydr. Res.*, **226**, 239—246 (1992).
37) Kulicke W. M., Lettau A. I., Thielking H., *Carbohydr. Res.*, **297**, 135—143 (1997).