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Physicochemical characterization of a high molecular weight bioactive β-D-glucan from the fruiting bodies of *Ganoderma lucidum*

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ABSTRACT

A purified polysaccharide coded as GLP20 was obtained by precipitating a hot-water extract from *Ganoderma lucidum* fruiting bodies with 20% (V/V) ethanol. Its total carbohydrate content was 95.9%. Structural analysis showed that GLP20 was a β-(1 → 3)-linked D-glucan with a (1 → 6)-β-D-glucopyranosyl side-branching unit on every third residue. Cell culture study revealed that GLP20 can significantly increase NO production of RAW264.7 macrophages. The analysis of light scattering and high performance size exclusion chromatography (HPSEC) showed that the molecular weight and polydispersity of GLP20 was 3.75×10^6 Da and 1.36, respectively. GLP20 had a rigid chain conformation in aqueous solution. A conformation transition occurred in the alkaline solution with NaOH concentration larger than 0.15 M. The transition from ordered structure to single chain happened when GLP20 was heated above 135 °C in water solution and was irreversible as demonstrated by differential scanning calorimetry (DSC). GLP20 existed as random coils in DMSO.

1. Introductions

The mushroom *Ganoderma lucidum* is a member of the fungus family (lamellae basidiomycete of the family Polyporaceae) and known as 'Lingzhi' in China and 'Reishi' in Japan. It has played an important role in Chinese traditional medicine for more than 4000 years (Zhang et al., 2010). Recent studies demonstrate that polysaccharides extracted from the fruit bodies, mycelia, and spores of *G. lucidum* can promote the function of macrophages, B cells, T cells as well as dendritic cells (Bao, Wang, Dong, Fang, & Li, 2002; Li, Fang, & Zhang, 2007; Meng et al., 2011; Shao, Dai, Xu, Lin, & Gao, 2004; Zhang, Tang, Zimmerman-Kordmann, Reutter, & Fan, 2002). There have been more than 200 polysaccharides isolated and purified from the fruiting bodies, spores, mycelia and cultivation broth of *Ganoderma* (Huie & Di, 2004). Most polysaccharides are heterosaccharides with different combinations of glucose, mannose, galactose, xylose, fucose as well as arabinose, and (1 → 3)-, (1 → 4)-, (1 → 6)-α, β-glucans are also identified (Miyazaki & Nishijima, 1981; Nie, Zhang, Li, & Xie, 2013; Sone, Okuda, Wada, Kishida, & Misaki, 1985; Wasser, 2002).

Comparing with other biopolymers such as proteins and nucleic acids, polysaccharides have the greatest potential for structural variability (Wasser, 2002), which results in the difficulties for establishing the structure-activity relationship for polysaccharides (Liu & Wang, 2006). It is generally accepted now that the branched (1 → 3, 1 → 6)-β-D-glucans play an important role in enhancing the antitumor and immunomodulatory effects, and a possible mechanism for their action is *via* binding to β-glucan specific receptors on innate immune cells, such as monocytes or macrophages, which might lead to subsequent activation of adaptive immunity (Akramiene, Kondrotas, Didziapetriene, & Kevelaitis, 2007; Rop, Mlcek, & Jurikova, 2009; Saito et al., 1991). These kinds of polysaccharides were reported to form higher ordered structures such as triple helices in aqueous solution. Several studies suggested that the higher ordered structures are responsible for the mentioned activities of (1 → 3, 1 → 6)-β-D-glucans (Falch, Espevik, Ryan, & Stokke, 2000; Yanaki et al., 1983; Zhang, Li, Xu, & Zeng, 2005). Such examples included lentinan and schizophyllan which have been accepted as commercial carbohydrate antitumor drugs (Daba & Ezeronye, 2004). The major bioactive *Ganoderma* polysaccharides species

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are branched (1 → 3)-β-D-glucan moiety (Sone et al., 1985). These structures are described as β-(1 → 3)-D-glucopyronans with 1–15 units of β-(1 → 6) monoglucosyl side chains (Russell & Paterson, 2006). However, the molecular size and branching degree of the glucan were found to influence its biological activities, as well as the conformation which could affect the bioactivities of the glucan (Sletmoen & Stokke, 2008).

In the present study, a high molecular weight β-D-glucan with demonstrated activity on stimulating macrophage cells was purified from the fruiting bodies of *G. lucidum* by a simple processing method. The detailed structure and conformational characteristics including the conformation transitions are reported. This work will offer useful information on the advanced structural features of *Ganoderma* polysaccharides, and will be helpful for further studying its activity.

2. Materials and methods

2.1. Polysaccharide isolation

Fruiting bodies of *G. lucidum* were cultivated by the Institute of Edible Fungi (IEF), Shanghai Academy of Agricultural Sciences (SAAS). Chemicals used in this study were analytical grade unless otherwise specified.

Air-dried fruiting bodies were ground into small pieces for polysaccharide extraction. 1500 g *G. lucidum* were extracted twice with 10 volumes of distilled water for 2 h at 100 °C. The combined aqueous extracts were filtrated and concentrated to 100 mL using vacuum evaporator. After being centrifuged at 6000 × *g* for 20 min, the supernatant was collected, followed by precipitation in 20% (v/v) ethanol at 25 °C. The ethanol aqueous system was kept at 4 °C overnight and the precipitate was collected by centrifuging (6000 × *g*, 15 min). The precipitate was rinsed with 30% ethanol for three times and freezing dried to yield the polysaccharide fraction GLP20.

2.2. Chemical and monosaccharide composition analysis

GLP20 (2 mg) was hydrolyzed with 2 M trifluoroacetic acid (TFA) at 110 °C for 3 h, and the monosaccharide composition was determined by high performance anion exchange chromatography (HPAEC) using a Dionex LC30 equipped with a CarboPac™ PA20 column (3 mm × 150 mm). The column was eluted with 2 mM NaOH (0.45 mL/min) and the monosaccharides were monitored using a pulsed amperometric detector (Dionex). Monosaccharide components were determined using D-Gal, D-Glc, D-Ara, L-Fuc, L-Rha, D-Man, and D-Xyl standards.

Content of total sugar was determined using the phenol–sulfuric acid method (DuBois, Gilles, Hamilton, Rebers, & Smith, 1956). The total protein contents were determined by analyzing the nitrogen content using NA 2100 protein analyzer (ThermoQuest, CE Instruments) and a conversion factor of 6.25 was used for the protein content calculation. All the measurements were repeated three times.

2.3. Methylation and GC–MS analysis

Methylation analysis of GLP20 was conducted according to the method of previous reports (Guo, Cui, Wang, & Christopher Young, 2008). The methylated polysaccharide was then converted into partially methylated alditol acetates (PMAA) by hydrolysis, reduction with $NaBD_4$, and acetylation, followed by linkage analysis using a GCQ ion trap GC–MS system (Thermo-Quest Finnigan, San Diego, CA) equipped with a SP-2330 (Supelco, Bellefonte, PA) column (30 m × 0.25 mm, 0.2 μm film thickness, temperature programmed from 160 to 210 °C at 2 °C/min, and then 210 to 240 °C at 5 °C/min, helium flow rate 1 mL/min). The individual peaks of the PMAA and fragmentation patterns were identified by their mass spectra and relative retention time in GC. The percentage of methylated sugars was estimated as ratios of the peak areas.

2.4. NMR spectroscopy

In order to improve the solubility of GLP20, ultrasonic treatment was performed using a JY-99 II ultrasonic reactor (Ningbo Xin Zhi Biotechnology Co., Ltd., China) of 20 kHz frequency and 1200 W maximum output power. 50 mL GLP20 solution with the concentration of 2 mg/mL was treated at 900 W for 2 h. The sample tube was surrounded with ice-water during treatment and the maximum temperature was less than 50 °C. The ultrasonic treated solution was freeze dried for NMR studies. Exchange protons were removed by dissolving GLP20 in D_2O, and subsequent lyophilization. This exchange process was repeated twice. High-resolution ^1H and ^{13}C NMR spectra were recorded in a mixed solvent, Me_2SO-*d6*/D_2O (6:1) (30 mg/mL), at 500.13 and 125.78 MHz, respectively, on a Bruker ARX500 NMR spectrometer operating at 60 °C. Chemical shifts are reported in ppm, using the internal Me_2SO signal (δ_H = 2.50 ppm) for ^1H and the internal Me_2SO signal (δ_C = 39.5 ppm) for ^{13}C as references. Homonuclear ^1H—^1H correlation spectroscopy (COSY, TOCSY) and heteronuclear ^1H—^{13}C correlation experiments (HMQC, HMBC) were run using the standard Bruker pulse sequence.

2.5. Cell culture and nitric oxide quantitation

RAW264.7, a mouse macrophage cell line, was incubated in DMEM medium (Gibco-Invitrogen Company, Carlsbad, CA, USA) containing 10% fetal calf serum (FCS), 100 U/mL penicillin and 100 μg/mL streptomycin at 37 °C in a 5% CO_2 atmosphere. Nitrite accumulation was used as an indicator of NO production in the medium as previously described (Ji et al., 2007). Cells (5×10^5 cells/mL) were dispensed into 96-well plates and stimulated with different samples for 48 h. Supernatants were then collected and mixed with 0.5 vol Griess reagent [1% (w/v) sulfanilamide, 0.1% (w/v) naphthylethylenediamine dihydrochloride, 2% (v/v) phosphoric acid] and incubated at room temperature for 10 min. Nitric oxide production was determined by comparing the absorbance at 543 nm against a standard curve generated using $NaNO_2$. Lipopolysaccharide (LPS, 1 μg/mL) was used as positive control in stimulating macrophage cells producing NO assay.

2.6. High performance size exclusion chromatography analysis

The solutions of GLP20 (0.5 mg/mL) in 0–1 M NaOH were prepared. Polydispersity and intrinsic viscosity were determined by high performance size exclusion chromatography (HPSEC) equipped with multiple detectors: a differential pressure viscometer (DP) for viscosity determination; a refractive index detector (RI) and a UV detector for concentration determination; a right angle laser light scattering detector (RALLS) and a low angle laser light scattering detector (LALLS) for direct molecular determination (Viscotek, tetra detector array from Malvern company). The detectors were calibrated with pullulan standards (P-100, JM Science, Inc., NY, USA). The chromatographic system comprised of a Shimadzu SCL-10Avp pump and automatic injector (Shimadzu Scientific Instruments, Inc., Columbia, MD 21046, USA). The columns were AquaGel series, PAA-M, PAA-M and PAA-203 in series (Poly-Analytic Inc., Canada). The degassed 0.1 M $NaNO_3$ containing 0.03% (w/w) NaN_3 was used as an eluent at a flow rate of 0.6 mL/min. The columns, viscometer and RI detector were maintained at 40 °C.

2.7. Light scattering

A 35 mW helium neon laser (Melles Criot Laser Group, Carlsbad, CA, USA) with wave length of 632.8 nm was focused on to a precision cylindrical cell (quartz, diameter 25 mm) containing a sample solution. Both static and dynamic measurements were conducted using a Brookhaven light scattering instrument including a precision Goniometer, a photomultiplier and a 128-channel BI-9000AT digital autocorrelator (Brookhaven Instruments, Holtsvile, NY, USA). Pure benzene at 25 °C was used to calibrate the apparatus. The test sample solutions were purified by filtration through nylon filters of pore size 0.45 μm. Both static and dynamic measurements were carried out in the angular range of 30°–140° and data was extrapolated to zero degree to yield desired parameters. The refractive index increment, dn/dc, was determined as 0.151 and 0.042 mL/g for GLP20 in water and DMSO, respectively. For dynamic measurement, the hydrodynamic radius (R_h) was calculated by applying Stokes–Einstein relation for sphere particles particles: $D = kT/6\pi\eta R_h$, where T is absolute temperature, k is the Boltzmann constant, and η is the solvent viscosity. The static light scattering allowed the determination of weight average molecular weight (M_w), radius of gyration (R_g).

2.8. Differential scanning calorimetry (DSC)

Thermal analyses were performed on a 2920 modulated DSC (TA Instruments, New Castle, DE, USA). Weigh about 3.0–3.5 mg dry matter of the GLP20 into a high volume stainless steel DSC pan, then the reagents were added into the pan to disperse the sample (5%, w/w). The sample dispersions were sealed hermetically and stored at 25 °C overnight. The samples were heated from 5 °C to 180 °C at a heating rate of 10 °C/min and cooled to 5 °C at 10 °C/min. The reported values are means of triplicates.

3. Results and discussions

3.1. Isolation, chemical and monosaccharide analysis of GLP20

Most polysaccharides of *G. lucidum* reported in the literatures were purified by columns such as DEAE cellulose chromatography and Sephadex series size exclusion chromatography (Jiang, Sun, He, & Shao, 2012; Ye et al., 2008, 2011; Zhao, Dong, Chen, & Hu, 2010). This process is tedious and difficult to obtain sufficient amount of purified polysaccharides for further investigation. In the present study, a purified polysaccharide coded as GLP20 was obtained by ethanol precipitation of hot water extract from the *G. Lucidum* fruit bodies. The yield was about 0.37% (w/w) on the basis of the dry weight of fruiting bodies. The purified polysaccharide contained 95.9% total sugar with no protein detected. The monosaccharide composition analysis of GLP20 indicated that it was a homoglucan. Chang and Lu (2004) reported a GLG fraction obtained by precipitation in 20% of ethanol. This fraction was further purified using gel filtration chromatography to get a β-D-glucan. In the current study, the purified homoglucan was obtained by a simple ethanol precipitation followed by three times of rinse with 30% ethanol. We provided a simple method to purify glucans from the extracts of *G. lucidum*.

3.2. Methylation analysis

Methylation analysis results indicated GLP20 was composed of nonreducing-end D-glucopyranosyl, (1 → 3)-linked D-glucopyranosyl, and (1 → 3, 1 → 6)-linked D-glucopyranosyl (branch point) moieties in a ratio 1.00:2.07:1.01. The result indicated GLP20 was an O-6-branched (1 → 3)-D-glucan.

3.3. NMR spectroscopy

In order to increase the solubility of the polysaccharides and yield high signal to noise ratio, polysaccharides with relatively lower molecular weight, which were generated using ultrasonic treatment, were used for the NMR analysis. The solubility was improved and the result of methylation analysis on ultrasonic treated sample was the same as the original sample, which indicated that the ultrasonic treatment did not change the primary structure of this polysaccharide.

The mixture of Me_2SO-*d6*/D_2O (6:1) was used as the solvent of the polysaccharide to obtain high-resolution signals in NMR spectroscopy (Tada, Adachi, Ishibashi, & Ohno, 2009). The 1D ^1H and ^{13}C NMR spectra are shown in Fig. 1. The anomeric signals at δ 4.5 ppm, δ 4.2 ppm (Fig. 1A) and δ 102.959 ppm (Fig. 1B) indicated a β configuration for glucopyranosyl units. Four signals were detected in the anomeric region (δ 4.2–4.7 ppm) in the ^1H NMR spectrum. Three of them were overlapped at δ 4.5 ppm and the other well-resolved doublet peak was observed at δ 4.2 ppm. The four sugar residues were arbitrarily labeled as A, B, C and D as described in Fig. 1A. All ^1H resonances were assigned based on COSY (Fig. 1C) and TOCSY (data not shown).

Two signals were shown in the anomeric region (δ 95–110 ppm) of the ^{13}C NMR spectrum (Fig. 1B). Based on the cross-peaks in the HMQC spectrum (Fig. 1D), the resonance at δ 102.861 ppm was assigned to residues A, B and C, and the other peak at δ 102.959 ppm was assigned to residue D. According to the data from HMBC (data not shown), the signals at δ 86.53, δ 86.06 and δ 85.74 ppm (Fig. 1B) arose from the substituted C-3 of glucose in units A, B and C, respectively, which is in agreement with previous literature (Tada et al., 2009). In addition, the combination of HMQC and HMBC experiments allowed the complete assignment of the ^{13}C spectrum. The ^1H and ^{13}C NMR spectral assignments of GLP20 are summarized in Table 1 based on the COSY, TOCSY, HMQC and HMBC spectra. Thus, the repeating unit of GLP20 was proposed as a (1 → 3)-β-D-glucan backbone with a (1 → 6)-β-D-glucopyranosyl side-branching unit on every third residue as described in Fig. 2. This repeat unit is different with some β-D-glucans isolated from the fruit bodies and spores of *Ganoderma* species (Dong et al., 2012; Miyazaki & Nishijima, 1981; Nie et al., 2013), which might result from the differences of materials and extract methods.

3.4. Effect of GLP20 on nitric oxide production in macrophages

Macrophages are the first line of host defence against bacterial infection and cancer growth (Fidler & Kleinerman, 1993; Verstovsek, Maccubbin, Ehrke, & Mihich, 1992), thus play an important role in the initiation of adaptive immune responses (Zirk, Hashmi, & Ziegler, 1999). Nitric oxide (NO) is one of the cytokines released by stimulating agents and is related to cytolytic function of macrophages against a variety of tumors (Hibbs, Taintor, & Vavrin, 1987; Li, Kilbourn, Adams, & Fidler, 1991). Therefore, macrophage-derived NO synthesis could contribute to the antitumor immune response *in vivo* (Yim, Bastian, Smith, Hibbs, & Samlowski, 1993). As shown in Fig. 3, the NO production of RAW264.7 cells treated with GLP20 (50, 200 and 500 μg/mL) for 48 h increased significantly comparing with the untreated controls. This indicated that GLP20 had obvious effect on stimulating macrophages to release NO.

3.5. Molecular characterization analysis

It has been reported that the family of β-(1 → 3)-glucans exhibited triple helical structures in nature at room temperature, leading to many special properties such as high viscosity, excellent tolerance to a broad range of pH and temperature in aqueous solution

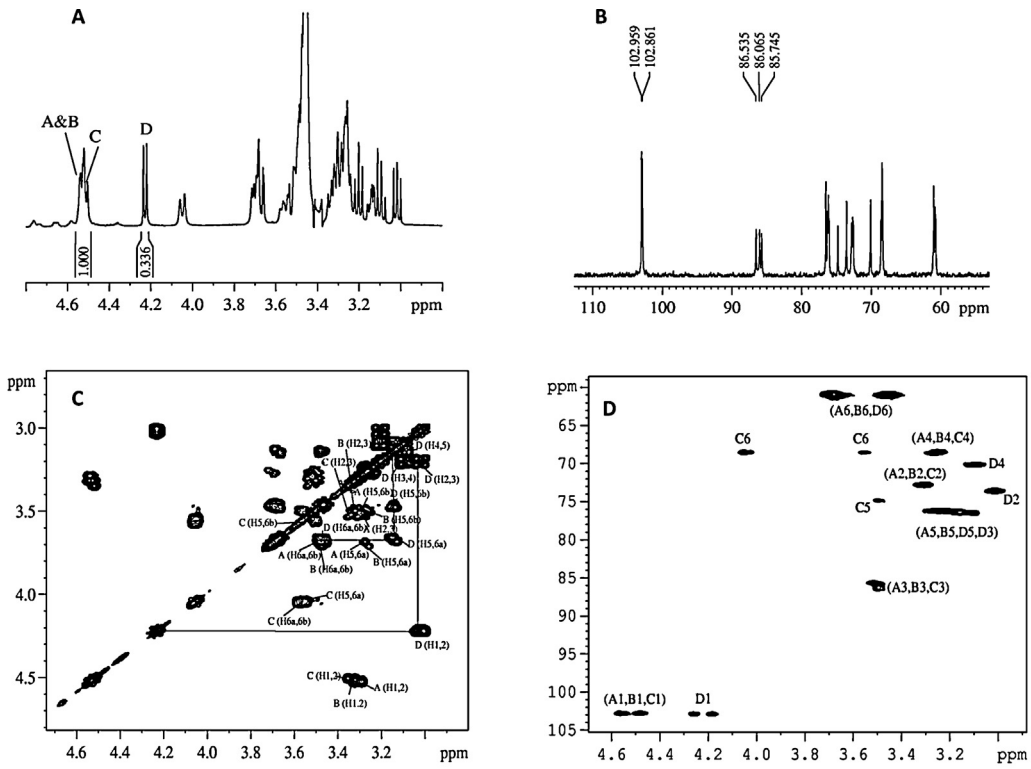

Fig. 1. NMR spectra of GLP20 (solvent: mixture of DMSO-*d6* with D_2O at the ratio of 6 to 1). (A–D) represent ^1H NMR spectrum, ^{13}C NMR spectrum, COSY spectrum and HMQC spectrum at 60 °C, respectively.

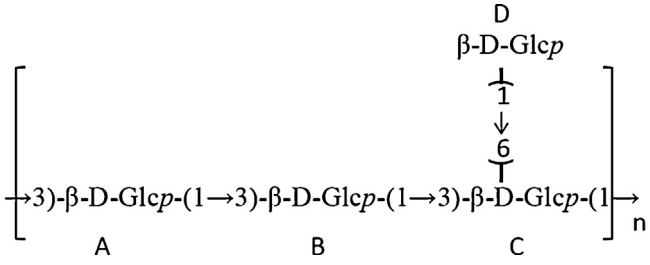

Fig. 2. Structure of GLP20 obtained from *G. lucidium*. Glc*p*: glucopyranose.

(Xu, Wang, Cai, & Zhang, 2010). In order to investigate the molecular characteristics of GLP20, HPSEC coupled with multiple detectors and light scattering analysis were carried out. As shown in Fig. 4A, a main peak was observed in the chromatogram for GLP20 in water solution. The polydispersity was 1.36 indicating a relative narrow distribution of M_w. GLP20 also exhibited high intrinsic viscosity ($[\eta]$ = 11.55 dL/g). Based on static and dynamic light scattering results, the M_w, the radius of gyration (R_g) and the hydrodynamic radius (R_h) for GLP20 in different solvents were summarized in Table 2. The M_w of GLP20 in 0.9% NaCl was 3.75×10^6 g/mol. M_w dependence of $[\eta]$ for GLP20 is illustrated in Fig. 5 on the basis of

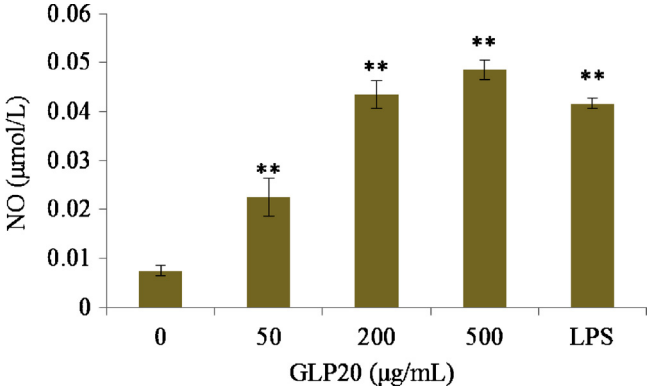

Fig. 3. Effect of GLP20 on nitrite production by macrophages. RAW264.7 cells were treated with different concentrations of GLP20 or LPS (1 μg/mL) for 48 h and culture supernatants were analyzed for nitrite production. Values are the means ± S.D. of triplicate determinations. $^{**}P < 0.01$, significant difference from the control group.

HPSEC data. Mark–Houwink equation for the glucan in 0.1 M $NaNO_3$ aqueous solution is

$$[\eta] = 1.78 \times 10^{-4} M_w^{0.83} \quad (\mathrm{cm^3\ g^{-1}})$$

Table 1
Chemical shifts (ppm) of ^1H and ^{13}C NMR signals for GLP20 recorded in DMSO-*d6*/D_2O (6:1) at 60 °C.

Sugar residue	H1/C1	H2/C2	H3/C3	H4/C4	H5/C5	H6a/C6	H6b
→ 3)-β-D-Glcp-(1 →	4.52	3.29	3.49	3.26	3.27	3.68	3.47
A	102.86	72.85	86.53	68.4	76.11	60.73	
→ 3)-β-D-Glcp-(1 →	4.52	3.32	3.49	3.25	3.26	3.71	3.47
B	102.86	72.69	86.06	68.45	76.22	60.88	
→ 3,6)-β-D-Glcp-(1 →	4.50	3.35	3.53	3.27	3.50	4.05	3.55
C	102.86	72.83	85.74	68.56	74.91	68.50	
β-D-Glcp-(1 →	4.22	3.02	3.19	3.10	3.14	3.66	3.48
D	102.95	73.55	76.35	70.22	76.50	61.01	

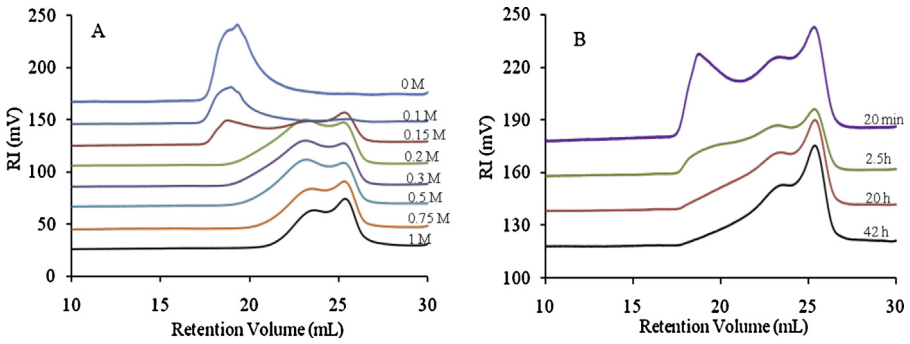

Fig. 4. Molecular size distribution profiles of samples treated with different concentrations of NaOH (0–1 M) and for different time. (A) The HPSEC elution curves of GLP20 treated in 0–1 M NaOH at room temperature for 20 min, then neutralized at pH = 7. (B) The HPSEC elution curves of GLP20 treated in 0.15 M NaOH for 20 min, 2.5, 20 and 42 h, respectively, then neutralized at pH = 7.

Table 2
Molecular characterizations of the GLP20 in different solvents.

Sample	$M_w \times 10^{-5}$ (g/mol)	R_g (nm)	R_h (nm)	ρ (=R_g/R_h)	$M_{w(0.9\%\ NaCl)}/M_{w(DMSO)}$
GLP20 in 0.9% NaCl	37.5 ± 2.4	135.7 ± 6.1	67 ± 1.2	2.03	2.8
GLP20 in DMSO	13.5 ± 0.26	61.7 ± 1.8	41 ± 2.3	1.50	

Usually, the exponent α of stiff chain polymer is higher than 0.8. The exponent α value of 0.83 indicates the stiff chain conformation of GLP20 in aqueous solution.

In a given polymer solution, the ratio of the radius of gyration to the hydrodynamic radius ($\rho = R_g/R_h$) depends on its chain architecture and conformation. It is reported that ρ is about 0.7–0.8 for a uniform sphere, 1.3–1.8 for a linear flexible random coil chain, and that is more than 2.0 for an extended rigid chain (Wang & Cui, 2005; Wang et al., 2009; Xu et al., 2010). The ρ (ratio of R_g to R_h) was 2.03 for GLP20 in 0.9% NaCl solution (Table 2), implying that GLP20 had a considerable rigid chain conformation in aqueous solution. Whereas the ρ value of 1.5 showed that GLP20 existed as random coils structure in DMSO. The value of M_w for GLP20 in 0.9% NaCl is about 3 times of that in DMSO suggesting that the predominant species of GLP20 exists as trimers in aqueous solution, whereas they dissociate into single-chain in DMSO. This result is in agreement with the same type of β-D-glucans such as lentinan (Zhang, Li, Wang, Zhang, & Cheung, 2011).

It was reported that the certain concentration of NaOH will cause the "denaturation" of the triple helical structure of β-(1 → 3)-linked glucan by breaking the hydrogen bonds (Sletmoen & Stokke, 2008). The triple helix-coils transition of lentinan occurred in the narrow ranges of NaOH concentration (0.05–0.08 M) (Zhang, Zhang, & Xu, 2004). For scleroglucan, the triplex dissociated to random coils in the alkaline solution with NaOH concentration in the range of 0.25–0.35 M (Kitamura et al., 1996). In order to test the conformation transition of GLP20 in alkaline solution, the polysaccharides dissolved in different concentrations of NaOH were analyzed by HPSEC. According to Fig. 4A, no "denaturation" of the ordered structure was observed in the solution of 0.1 M NaOH as the chromatogram was identical with that in water. "Denaturation" began to occur in 0.15 M NaOH solution since the original peak coexisted with the unfolded one. The HPSEC elution curves for GLP20 in NaOH solutions with concentration larger than 0.2 M revealed that the ordered structures disappeared after 20 min, which proved that the unfolding process was fast. After "denaturation", two overlapped peaks existed in the solution. With the increasing of NaOH concentration (Fig. 4A) or extending of treating time (Fig. 4B), a shift of the peak from low to high retention volume appeared slowly, but they always coexisted with each other during the storage in this experiment. This might be resulted from the denaturation or degradation of GLP20 during the process and needs to be verified in future studies.

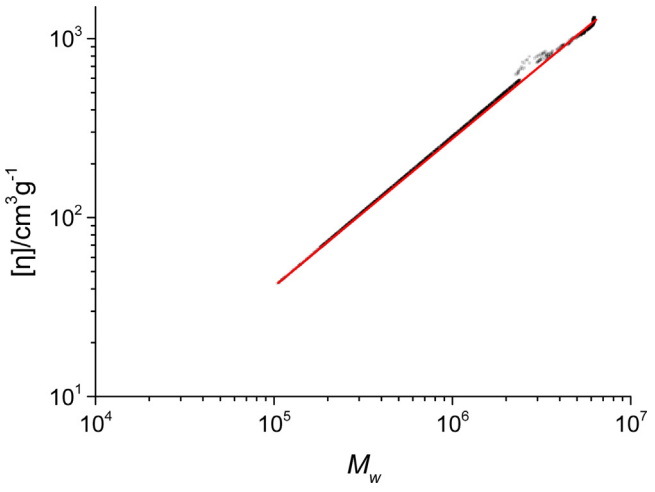

Fig. 5. The double-logarithmic plot of [η] against M_w for GLP20 in 0.1 M $NaNO_3$ at 40 °C.

3.6. DSC measurements of GLP20 in water and DMSO

DSC is a useful technique for obtaining thermodynamic information on the conformational transition process of polysaccharides and proteins. Endothermic peaks during the conformational transition from DSC measurement indicate the melting of structural domains (Lazaridou, Biliaderis, Micha-Screttas, & Steele, 2004).

The DSC heating and cooling curves for GLP20 in water and DMSO are shown in Fig. 6A. There was a significant endothermic peak for 5% water dispersion of GLP20 during heating from 5 to 160 °C. The onset melting temperature was about 139.43 °C, and the transition temperature was about 143.35 °C. According to previous reports, there was a triple helix-single coil transition at higher temperature for several kinds of (1 → 6) branched (1 → 3)-β-D-glucans, and the transition temperatures were correlated to the degree of branching for these glucans (Kitamura et al., 1990). It was also proved that scleroglucan triple helix dissociated to random coils

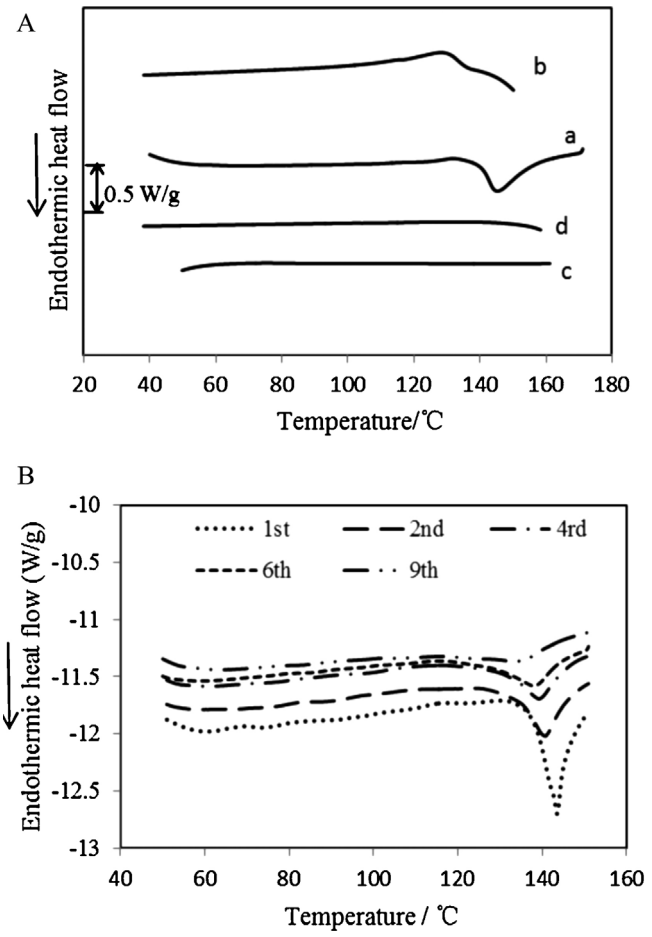

Fig. 6. DSC heating and cooling curves of GLP20 dispersion (5%, w/w). (A) DSC heating and cooling curves of GLP20 dispersion in water (a, b) and DMSO (c, d) during heating and cooling at a rate of 10 °C/min. (B) DSC curves of GLP20 dispersion (5%, w/w) in water during heating for nine times at a rate of 1 °C/min.

when heated above 135 °C in aqueous solution (Kitamura & Kuge, 1989). Therefore, GLP20 could be dissolved as an ordered triple-helical structure in water which melted at 139 °C. In contrast, the appearance of exothermic peak upon cooling is considered as the reformation of hydrogen bonds.

The DSC curve for GLP20 in DMSO was different from the water dispersion. No visible endothermic or exothermic peaks were observed during heating and cooling as shown in Fig. 6A(c) and (d), suggesting that both processes did not involve any change in hydrogen bonds, which indicated that GLP20 was random coils in DMSO. This observation is in agreement with literature reports for lentinan (Zhang, Li, Zhou, Zhang, & Chen, 2002) and schizophyllan (Kitamura & Kuge, 1989).

As shown in Fig. 6B, the GLP20 solution which had been melted at high temperature was rescanned for several times after being cooled to 5 °C. The endothermic peaks shifted to lower temperature gradually with the increasing times of rescanning, and the endothermic enthalpy decreased gradually, suggesting that the denatured samples could not form new ordered conformation immediately upon cooling. This indicated that the structure transition for GLP20 in water dispersion at a temperature higher than 135 °C was irreversible within the experiment period. This is consistent with other (1 → 3, 1 → 6)-β-D-glucans such as lentinan (Wang, Xu, & Zhang, 2008) and schizophyllan (Yanaki, Tabata, & Kojima, 1985).

4. Conclusions

A high molecular weight β-(1 → 3)-D-glucan with (1 → 6)-β-D-glucosyl branches was obtained from the fruiting bodies of *G. lucidum* by a simple ethanol precipitation method. The detailed structure analysis showed that GLP20 had a regular repeat unit with degree of branching rate at 1/3, which is different from most reported structures of glucans from *Ganoderma* species due to the differences on materials and extract methods. This β-D-glucan was able to stimulate macrophage cells to release NO on a dose response manner. It existed as a rigid chain conformation in water solutions like an ordered trimer. A conformation transition occurred in alkaline solution when NaOH concentration was larger than 0.15 M. DSC measurements revealed that the conformation transition for GLP20 in water solution happened when it was heated above 135 °C.

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