Structure characterization of a novel neutral polysaccharide isolated from Ganoderma lucidum fruiting bodies

Deng Pan a, Linqiang Wang b, Congheng Chen a, Baosong Teng a, Chendong Wang a, Zhixue Xu a, Bingwen Hu b,⇑, Ping Zhou a,⇑

a State Key Laboratory of Molecular Engineering of Polymers, Fudan University, Shanghai 200433, PR China
b Shanghai Key Laboratory of Magnetic Resonance, Department of Physics, East China Normal University, Shanghai 200062, PR China

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ABSTRACT
Ganoderma lucidum (G. lucidum) is a mushroom which has been used for health promotion for a long time in China. In the present work a neutral hetero-polysaccharide, named FYGL-1, was isolated from FYGL which was reported previously capable of antihyperglycemia in vivo for further detailed chemical structure investigation. The results of monosaccharide composition and GPC analysis indicated that FYGL-1 consisted of galactose, rhamnose and glucose in mole ratio of 1.00:1.15:3.22 with a molecular weight of 78 kDa. The detailed structure of FYGL-1 was characterized by periodate oxidation, Smith degradation, methylation analysis, along with FT-IR, GC, GC–MS, 1D1H and 13C NMR and 2D NMR (HSQC, COSY, NOESY and TOCSY). Based on the analysis of the results, the structure of the repeating unit of FYGL-1 was established as:

\[ \rightarrow 2\)-β-L-Rhap-(1→6)-α-D-Galp-(1→6)-α-D-GlcP-(1→3) 2
\[ \uparrow \uparrow \uparrow \]
\[ α-D-GlcP \quad α-D-GlcP \]

1. Introduction

Throughout history, many natural products have been used for the prevention and treatment of various human ailments. One example is Ganoderma lucidum (G. lucidum), a rot fungus, also known as “Lingzhi” in Chinese, “Reishi” in Japanese, “Youngzh” in Korean. G. lucidum is a mushroom which has been widely used as a tonic for promoting longevity and health in China and other Asian countries for more than 2000 years (Paterson, 2006; Sone et al., 1986). Recently, a great deal of work has been carried out on this fungus due to the fact that dozens of extracts isolated from them have shown obvious medicinal potencies on cancer, hypertension, oxidation, hypercholesterolemia, and immunological diseases (Huang et al., 2010; Trajkovic, Mijatovic, Maksimoviclvanic, Stojanovic, & Tufegdzic, 2009; Weng et al., 2009; Yeh, Chen, Yang, Chuang, & Sheu, 2010).

As important biologically active components in G. lucidum, polysaccharides and proteoglycans were reported to play many special roles. For instance, Guo et al. (2009) reported a water-soluble polysaccharide (GSG) extracted from the spores of G. lucidum. GSG was an effective inducer of MAPKs- and Syk-dependent TNF-α and IL-6 secretion in murine resident peritoneal macrophages. Additionally, in vivo administration of GSG potentiataed the ConA-induced proliferative response of splenocytes and induced the anti-tumour activity against Lewis lung cancer in mice, suggesting that GSG was an effective immuno-modulator and maybe a promising adjuvant remedy for anti-tumour therapies. Chen, Xie, Nie, Li, and Wang (2008) reported a water-soluble protein-bound polysaccharide (PSG-1) extracted from the fruiting bodies of G. lucidum. PSG-1 showed the strong activity to scavenge the 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical and the superoxide anion radical, suggesting that PSG-1 could potentially be used as natural...
antioxidants. Yang, Wang, Xie, Sun, and Wang (2011) extracted a polysaccharide from G. lucidum (GLP) using boiling water. Effects of GLP on the activity of antioxidant enzymes including superoxide dismutases, catalase, glutathione peroxidases and reduced glutathione, and the level of lipid peroxidation of pancreas were studied using STZ-induced diabetic rats. Result showed that GLP could effectively reduce the oxidative injury and inhibit the apoptosis by increasing antioxidant enzyme activity and modifying bcl-2/bax ratio. Li, Fang, and Zhang (2007) reported that GLP-2 isolated from the submerged culture broth of a basidiomycete G. lucidum could significantly enhance the T and B lymphocyte proliferation and the antibody production in vivo. Animal test of GLP-2 also showed a hepatoprotective activity in liver injured mice induced by lipopolysaccharide. Furthermore, some other recent research has demonstrated that the polysaccharides and proteoglycans extracted from G. lucidum could promote the release of serum insulin and decrease the plasma glucose level in vivo (Fatmawati, Shimizu, & Kondo, 2011; Jia et al., 2009; Meng et al., 2011). Because of important functions for health care, the extracts from G. lucidum have become popular dietary supplements even in western countries, with an annual global market value of over $1.5 billion (Sullivan, Smith, & Rowan, 2006). However, different G. lucidum strains may have different chemical compounds and potencies due to the differences in geographical distributions, growth conditions and substrates (Somajeh, Hasan, Hossain, Fateme, &Mohammad, 2010).

In insulin signalling pathway, there is one important phosphatase, protein tyrosine phosphatase 1B (PTP1B) considered to play an important role in the intracellular signal transduction process and metabolism (Asante & Kennedy, 2003). Therefore, searching for an effective inhibitor of PTP1B activity has been considered worthwhile recently for developing a promising insulin-sensitive drug for the prevention and treatment of metabolic disorders (Chen, Zhang, & Huang, 2010). Previously, we successfully isolated a bioactive extract, named FYGL, from the fruiting bodies of G. lucidum (Teng, Wang, Yang, et al., 2011; Teng, Wang, Zhang, et al., 2011). FYGL is capable of inhibiting PTP1B activity and antihyperglycemia in vivo. However, structural information such as sugar residue linkages, branches, and substitute groups in FYGL is still unclear due to its complicated chemical structure. For elucidation of chemical structure, FYGL was further fractionated by anion-exchange chromatography, and a novel neutral polysaccharide, named FYGL-1, was isolated. Therefore, the present study attempted to characterize the chemical structure of FYGL-1 in detail by FTIR, GC, GC–MS, methylation analysis, periodate oxidation, Smith degradation, 1D and 2D NMR, as a prelude to establish the structure–function relationships.

2. Materials and methods

2.1. Materials and chemicals

All the dried fruiting bodies of G. lucidum grown in north-eastern China were purchased from Leiyunshang Pharmaceutical Co. Ltd. (Shanghai, China). Standard monosaccharides and dextrans with different molecular weights were purchased from Sigma (Shanghai, China). DEAE-52 cellulose, Sephadex G-75 and Sepharose CL-4B were from Whatman Co. (Shanghai, China). Dimethyl sulphoxide (DMSO) and methyl iodide (CH₃I) from Sinopharm Co. Ltd. (Sichuan, China). Trifluoroacetic acid (TFA), sodium periodate (NaIO₄), sodium iodate (NaIO₃) and other necessary reagents from Sinopharm Co. Ltd. (Shanghai, China) were of analytical grade and used without further purification.
successively; the crude extract (yield 4.5 g) was then selected. Subsequently, the crude extract was further fractionated using a Sephadex G-75 column (I.d. 26 × 80 cm) and the main fraction, named as FYGL (yield 1.54 g) was collected.

The quality control of FYGL was performed on an Agilent 1100 HPLC system (Milford, MA, USA) coupled to both UV and refractive index detectors, with a PL aquagel-OH mixed bed column and 0.05 mol/L Na2SO4 was used as the eluent with flow rate of 0.5 mL/min at 35 °C (data in Supporting information S1). There were three peaks detected at 17.8, 22.5 and 27.5 min by RI detector, and one peak detected at 22.5 min by UV at a wavelength of 254 nm as well. The peak detected at 31.6 min by RI was due to solvent. The ratio of the three peak areas detected by RI detector was 7.6:88.4:5.0 with error of ±3% on the repeats at least ten times for the amount required by the animal trials. The extraction process was performed with relatively high reproducibility.

2.4. Isolation of FYGL-1

Component analysis indicated that FYGL was composed of polysaccharide and protein, therefore in present work, FYGL was further fractionated using DEAE-52 cellulose column (I.d. 60 × 40 cm) and 0–0.3 M NaCl as eluents in a stepwise-gradient manner. Four fractions were separated from FYGL, and one of fractions, named FYGL-1 (yield of 5.8% referred to FYGL), was used for structure analysis in the present work.

2.5. Determination of homogenity and molecular weight of FYGL-1

The homogenity and molecular weight of FYGL-1 were determined by gel permeation chromatographic (GPC), using Sepharose CL-4B (Cheong, Jung, & Park, 1999). The molecular weight was calibrated with T-series Dextran (T-110, T-70, T-40 and T-10). A 0.1 M NaCl solution with flow rate of 0.5 mL/min was used as the eluent at 25 °C. Pre-swollen Sepharose CL-4B was washed twice with 0.3 M NaOH and three times with water, finally equilibrated with distilled water in a glass column (I.d. 50 × 1.8 cm) for 3 days. Standard dextrans T-110, T-70, T-40 and T-10 (10 mg) were passed through the Sepharose CL-4B column, and then the elution volumes were plotted against the logarithms of their corresponding molecular weights. The elution volume of FYGL-1 was then plotted in the same graph, and molecular weight of FYGL-1 was thus determined (data in Supporting information S2).

2.6. Monosaccharide and configuration analysis

Monosaccharides composition and their absolute configurations were analyzed on the basis of the literatures (Gerwig, Kamering, & Vliegenthart, 1978; Jones & Albersheim, 1972). Gas chromatography (GC) is an efficient method in determination of the saccharides for its excellent performance, rapidity and sensitivity (Huiea & Di, 2004). For monosaccharide analysis, a solution of 250 μL 0.625 M HCl in+(−)-2-butanol was added into the hydrolyzed product of FYGL-1, and kept at 80 °C for 16 h. The trimethylsilylated (TMS)-(−)-2-buty glycoside-derivatives of monosaccharide were prepared with N,O-bis (tri-methylsilyl) trifluoroacetamide (BSTFA) which were also analyzed by GC.

2.7. FTIR spectral analysis

For FTIR analysis of FYGL and FYGL-1, 3.0 mg sample were ground together with 20 mg potassium bromide (KBr) powder and pressed into pellet. FTIR spectrum was recorded within wave-number of 4000–500 cm⁻¹ with resolution of 4 cm⁻¹. Data is shown in the Supporting information S3-a and S3-b.

2.8. Methylation analysis

In order to determine the glycosyl linkages, FYGL-1 (5.0 mg) was methylated four times referred to the Needs’ method, using distilled DMSO and finely powdered dry NaOH (Needs & Selvendran, 1993). The vacuum-dried FYGL-1 (5 mg) was dissolved in DMSO (1.5 ml) and then methylated with a saturated NaOH/DMSO solution (1.5 ml) and CH3I (1 ml). The reaction mixture was extracted with chloroform, and the organic phase was washed with double-distilled water. Complete methylation was confirmed by the disappearance of −OH band (3200–3700 cm⁻¹) in FTIR spectrum. The permethylated FYGL-1 was hydrolyzed, reduced and acetylated similarly as mentioned in Section 2.6. After those procedures, the methylated alditol acetates were re-dissolved in chloroform and analyzed using a gas chromatograph-mass spectrum (GC–MS, Shimadzu GC-14A, Tokyo, Japan). The results are shown in Fig. 3.

2.9. Periodate oxidation and Smith degradation

Periodate oxidation and Smith degradation were performed to confirm the glycosidic linkages deduced from methylation analysis. FYGL-1 (10 mg) was oxidized with 0.015 M NaIO4 (25 ml) at

Fig. 2. GC chromatogram of monosaccharides composition in FYGL-1. (a) references, peaks from left to right: Rha, Fuc, Ara, Xyl, Man, Glc, Gal, GlcA, GalA; and (b) monosaccharides of FYGL-1, peaks from left to right: Rha, Glc, Gal.
4 °C in the dark. The extent of reaction was monitored daily by UV absorption at 224 nm till the absorption intensity reached the lowest (Dixon & Lipkin, 1954). After the oxidation was completed, the consumption of NaIO₄ was determined quantitatively by its UV absorption at 224 nm, meanwhile the production of formic acid was measured by titration with a standard sodium hydroxide solution (0.01 M). The excess periodate was destroyed by adding two drops of glycol, and then the solution was dialyzed, reduced, neutralized, dialyzed again and lyophilized to give the polyalcohol.

The elution profile of FYGL-1 was free of protein. Furthermore, no uronic acid was detected by m-hydroxydiphenol method with D-glucuronic acid as a standard. Phenol–sulphuric acid assay, used to determine the total sugar content, indicated that FYGL-1 contained 97% carbohydrate. All those results demonstrated that FYGL-1 was a neutral polysaccharide.

For monosaccharide and absolute configuration analysis, FYGL-1 was hydrolyzed, reduced and acetylated. The results from GC spectrum indicated that FYGL-1 was composed of D-galactose, D-glucose, and D-glucuronic acid, with a mole ratio of 1.00:1.15:3.22, as shown in Fig. 2.

FTIR spectrum of FYGL-1 exhibited absorption bands at 3390, 2928, 1452, 1384, 1047, 881, and 840 cm⁻¹ (data in Supporting information S3-b). The broad band at 3390 cm⁻¹ was characteristic of hydroxyl group and the weak band at 2928 cm⁻¹ was denoted the C–H stretching vibration. The absorptions at 1452 and 1384 cm⁻¹ were possibly attributed to the bending vibration of CH₂ group and stretching vibration of C–O–C group, respectively (Zou et al., 2010). The characteristic absorptions at 840 and 881 cm⁻¹ indicated that α- and β-configurations present simultaneously (Barker, Bourne, Stacey, & Whiffin, 1954). The absorption band of 1047 cm⁻¹ suggested that FYGL-1 consisted of pyranoside (Huang & Ning, 2010).

3.2. Linkage features of FYGL-1

Methylation analysis by GC–MS was employed to determine the glycosyl linkages of FYGL-1. As shown in Fig. 3 and summarized in Table 1, the results showed the presences of four types of linkages 2,3,4,6-Me₄-Glc, 2,4-Me₂-Gal, 3,4-Me₂-Glc and 3,4-Me₂-Rha in mole ratio of 1.5:1.2:1.0:1.3. The mole ratio of these residues agreed overall with the percentages of monosaccharides in FYGL-1unit of 1 described above. Therefore, it was suggested that the repeating unit of FYGL-1 consisted of 1,2,6-linked glucose, 1,2-linked rhamnose, 1-linked glucose and 1,3,6-linked galactose.

Periodate oxidation and Smith degradation of FYGL-1 were performed to confirm the glycosidic linkages deduced from methylation analysis. Based on the average molecular weight of monosaccharide residue of 180.5 which was deduced from the

### Table 1

<table>
<thead>
<tr>
<th>Peak No.</th>
<th>Methylated sugars</th>
<th>Primary fragments (m/z)</th>
<th>Molar ratio</th>
<th>Linkages</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2,3,4,6-Me₄-Glc</td>
<td>101, 161, 129, 145, 87, 45, 205</td>
<td>1.5</td>
<td>1-Glc</td>
</tr>
<tr>
<td>2</td>
<td>3,4-Me₂-Glc</td>
<td>101, 99, 129, 149, 208, 234</td>
<td>1.0</td>
<td>1,2,6-α-Glc</td>
</tr>
<tr>
<td>3</td>
<td>2,4-Me₂-Gal</td>
<td>129, 189, 87, 159, 233</td>
<td>1.2</td>
<td>1,3,6-α-Galp</td>
</tr>
<tr>
<td>4</td>
<td>3,4-Me₂-Rha</td>
<td>87, 129, 131, 189</td>
<td>1.3</td>
<td>1,2-β-Rhap</td>
</tr>
</tbody>
</table>

* 2,3,4,6-Me₄-Glc: 1,5-diacetyl-2,3,4,6-tetra-O-methyl-gulitol.
* The m/z of 43 and 117 of acetyl ion were detected in all glycosyl residues.
* The mass fragments are ordered in intensity decreased.

Fig. 3. GC–MS profile of methylated FYGL-1. Signals from 1–4 represent 2,3,4,6-Me₄-Glc, 2,4-Me₂-Galp, 3,4-Me₂-Glc and 3,4-Me₂-Rhap.
mole ratio of monosaccharide compositions in FYGL-1, it was concluded that a total of 1.16 mol of periodate was consumed and 0.36 mol of formic acid was liberated per mole of sugar residue. The fact that the consumption of periodate was two times more than the production of formic acid indicated that the glycosyl linkages of 1 → 2, 1 → 2,6, 1 → 4 and 1 → 4,6 might exist. The oxidized product was reduced and hydrolyzed, and GC analysis was performed to identify glycerol, and galactose in a mole ratio approximately 10:1 (data in Supporting information S4), indicating that the d-glucose and l-rhamnose were consumed during the oxidation. There were large amounts of glycerol production, indicating that there may exist a lot of hexapyranose with glycosyl linkages of 1 → 1, 1 → 6 and 1 → 2,6. The presence of galactose after Smith degradation implies that the d-galactose might be linked in the form of 1 → 3, 1 → 2,3, 1 → 2,4, 1 → 3,4, 1 → 3,6 or 1 → 2,3,4. No erythritol was noticed, indicating that there were no glycosyl linkages of 1 → 4 and 1 → 4,6 in the backbone of FYGL-1. The results from periodate oxidation and Smith degradation agreed with the expected results based on the linkage analysis of methylation.

3.3. Determination of the sugar residues and their sequence by NMR

Signals of FYGL-1 in 1D $^1$H and $^{13}$C NMR and 2D NMR (HSQC, COSY, NOESY and TOCSY) spectra were assigned as completely as possible, based on the monosaccharide analysis, linkage analysis and chemical shifts reported in the literature (Evelina et al., 2009; Jin et al., 2010; Soumitra, Indranil, Dilip, & Syed, 2006; Wang, Wei, & Jin, 2009). $^1$H NMR spectrum (Fig. 4a) contained four signals

![Fig. 4](https://example.com/fig4.png)

**Fig. 4.** Parts of 1D and 2D NMR spectra of FYGL-1 in D$_2$O. (a) $^1$H NMR spectrum; (b) $^{13}$C NMR spectrum; (c) $^1$H–$^{13}$C HSQC spectrum; (d) $^1$H–$^1$H COSY spectrum; (e) $^1$H–$^1$H TOCSY spectrum, and (f) $^1$H–$^1$H NOESY spectrum. The Arabic numerals and capital letters refer to atoms and sugar residues, respectively, as shown in Table 2.
Table 2
Assignments (ppm) of $^1$H and $^{13}$C NMR of FYGL-1$^a$.$^b$

<table>
<thead>
<tr>
<th>Sugar residues</th>
<th>H-1/C-1</th>
<th>H-2/C-2</th>
<th>H-3/C-3</th>
<th>H-4/C-4</th>
<th>H-5/C-5</th>
<th>H-6/C-6</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\alpha$-D-Galp-(1→A)</td>
<td>5.26/101.7</td>
<td>3.62/76.76</td>
<td>4.21/78.18</td>
<td>3.98/na$^a$</td>
<td>4.13/66.02</td>
<td>3.99/81.11</td>
</tr>
<tr>
<td>$\alpha$-D-Glc-(1→B)</td>
<td>5.18/102.8</td>
<td>3.80/77.43</td>
<td>3.99/69.6</td>
<td>3.56/76.61</td>
<td>3.76/63.14</td>
<td>3.95/75.04</td>
</tr>
<tr>
<td>$\alpha$-D-Glc-(1→C)</td>
<td>5.05/103.1</td>
<td>3.80/67.72</td>
<td>3.99/na</td>
<td>3.50/71.55</td>
<td>3.76/71.02</td>
<td>na/na</td>
</tr>
<tr>
<td>$\alpha$-D-Glc-(1→D)</td>
<td>4.48/103.8</td>
<td>3.35/75.15</td>
<td>3.50/74.51</td>
<td>3.72/67.18</td>
<td>3.77/na</td>
<td>1.24/19.29</td>
</tr>
</tbody>
</table>

$^a$ Bold data indicate the linkage positions.

$^b$ na: not assigned.

The conclusion of the sequences in the repeating unit was determined based on the cross-peaks of protons observed in NOESY as shown in Fig. 4f. The cross peak $\delta$ 5.05/4.21 was observed in NOESY (Fig. 4f). Since $\delta$ 5.05 corresponded to H-1 in residue C, and $\delta$ 4.21 to H-3 in residue A, it could be concluded that the residues A and C were connected to each other. Moreover, the cross-peaks 5.26/3.80, 5.26/3.95 and 5.05/3.80 were observed in NOESY (Fig. 4f). Since 5.26, 3.80, 3.95 and 5.05 corresponded to H-1 in residue A, H-2 in residue B, H-6 in residue B, and H-1 in residue C, respectively, it was concluded that residues A and B, residues A and D, and residues B and C were connected to each other.

Based on the experimental data presented above, including methylation analysis, periodate oxidation, Smith degradation and NMR characterization, etc. it was proposed that the residue sequence in the repeating unit was as: $\text{D} \rightarrow \text{A} \rightarrow \text{B}$

4. Conclusion

A neutral polysaccharide, named FYGL-1, was fractionated from a hypoglycemic extract of $G. lucidum$, FYGL, by DEAE-52 cellulose column chromatography. The molecular weight of FYGL-1 was about 78 kDa. Monosaccharide analysis revealed that FYGL-1 was a heteropolysaccharide composed of galactose, rhamnose and glucose residues in mole ratio of 1.00:1.15:3.22. Based on the analysis of methylation, periodate oxidation, Smith degradation, 1D and 2D NMR, the backbone structure of FYGL-1 consisted mainly of 1,2-linked-$\alpha$-L-Rhap, 1,3,6-linked-$\alpha$-D-Galp, 1,2,6-linked-$\alpha$-D-Glcp and 1-linked-$\alpha$-D-Glcp.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.foodchem.2012.05.071.