Structural characterization of LbGp1 from the fruits of Lycium barbarum L.

Zhongfu Wang, Yang Liu, Yujiao Sun, Qing Mou, Bo Wang, Ying Zhang, Linjuan Huang *

Educational Ministry Key Laboratory of Resource Biology and Biotechnology in Western China, Life Science College, Northwest University, Xi’an 710069, China

1. Introduction

Lycium barbarum L., a kind of multi-branched shrub, belongs to the genus Lycium of the family solanaceae. Red-coloured fruit of L. barbarum L. (also called Gouqizi or wolfberry), which has a series of functions such as 'nourishing both the kidney and liver, moistening the lungs, producing essence and enhancing vision', has been consumed as a popular functional food and medicine in Asian countries for at least 2000 years (Huang, Lin, Tian, & Ji, 1998). As a food, it is traditionally cooked with porridge or soup before consumption and also can be used to produce various healthy products and foods, including canned fruits, yogurts, beverages, jams, jellies and wines (He, Yang, Jiao, Tian, & Zhao, 2012).

So far, much research has been done on L. barbarum L. A large and growing body of studies have showed that L. barbarum fruits have a variety of beneficial effects on immunoregulation, anti-aging, lowering blood-sugar and blood-fat levels, anti-fatigue, male fertility-facilitation and so on (Chen, Lu, Srinivasan, Tan, & Chan, 2009; Huang, Zhang, Jiang, Kang, & Zhao, 2012). Research has shown that many of the world’s longest living people consume L. barbarum fruit on a daily basis, suggesting it may be one of the world’s most powerful anti-aging foods (Yu et al., 2005).

The growing interest in the nutritional role and chemotherapeutic properties of L. barbarum fruit has prompted detailed identification of its constituents. It is reported that wolfberries contain an abundance of health-promoting constituents such as vitamins, flavonoids, zeaxanthin, carotenoids, and polysaccharides (Qian, Liu, & Huang, 2004; Wang, Chang, & Chen, 2009). Among all of the bioactive substances, polysaccharides represent a type of main bioactive constituents. The polysaccharides from fruit of L. barbarum L. have been found to possess many health-promoting and medical effects, such as stimulating dendritic cell immunogenicity (Shen & Du, 2012), activating macrophage, improving the antioxidant and anti-aging ability (Wang, Chang, Stephen Inbaraj, & Chen, 2010; Wu, Guo, & Zhao, 2006) and inhibiting the proliferation of human hepatoma QG7703 cells (Zhang et al., 2005).

Numerous studies mainly focus on bioactivity of L. barbarum polysaccharides in recent years, and crude polysaccharides extracted from L. barbarum L. are widely used to investigate its biological activities. Chemical studies on L. barbarum polysaccharide, especially in the field of structure characterization, have lagged behind its bioactive studies, due to its structural complexity and the difficulties in its separation and purification. Traditionally,

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**Abbreviations:** HPGPC, high-performance gel permeation chromatograph; EI-MS, electrospray-ionization mass spectrometry; GC, gas chromatography; GC–MS, gas chromatography–mass spectrometry; FT-IR, Fourier transform infrared spectroscopy; TFA, trifluoroacetic acid; NaBH₃CN, sodium cyanoborohydride; Ara, arabinose; Gal, galactose; LBP, Lycium barbarum glycoconjugate polysaccharide; LbGp1, L. barbarum glycoconjugate polysaccharide; LbGp1-OL, the glycan of the LbGp1.

* Corresponding author. Tel.: +86 29 88303533; fax: +86 29 88303534.

E-mail address: huanglj@nwu.edu.cn (L. Huang).

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structure analysis of polysaccharide are carried out mainly by methylation analysis, partial acid hydrolysis combined with chromatography, mass spectrometry, nuclear magnetic resonance, Fourier transform infrared spectroscopy, etc. (Peng et al., 2012). In order to make full use of this functional food resource, it is necessary to investigate the structure and bioactivity of *L. barbarum* polysaccharide.

Several studies were performed to characterize the structure of polysaccharides isolated from the fruits of *L. barbarum*, however, the sugar compositions and structure features of polysaccharides from *L. barbarum* showed significantly different patterns due to the different separation methods. For example, an immunoactive glycoprotein (LbGP) from *L. barbarum* was reported to be composed of Ara, Gal, and Glc and 18 kinds of amino acids (Tian, Wang, & Feng, 1995). While *L. Barbarum* acidic polysaccharide LBP-1 was determined to be composed of Rha, Ara, Xyl, Gal, Man and GalA, further linkage analysis showed that it’s backbone was mainly composed of (1,5)-linkage arabinose, (1,4)-linkage galacturonic acid, (1,3,6)-linkage mannose (Zou, Zhang, Yao, Niu, & Gao, 2010). Despite the differences existed in the structural analysis of the polysaccharide, these studies have proved to providing an effective insight into understanding the structure–function relationships of polysaccharides existing in this very important medicinal and functional food, *L. barbarum*.

In our previous work (Huang et al., 1998), five water-soluble polysaccharides (designated as LbGP1–LbGP5) from *L. barbarum* were isolated and three of them (LbGP2, LbGP3, LbGP4) were structurally elucidated (Huang, Tian, & Ji, 1999; Huang, Tian, Qi, & Zhang, 2001; Peng & Tian, 2001). We also found that these purified polysaccharides had a variety of immunomodulatory effects especially activation of T cells, B cells, and dendritic cells (Qi et al., 2001), which showed medicine exploiting prospect.

The current study is focused on the structural analysis of polysaccharide LbGP1 fraction from *L. barbarum*. After purification, Structure of LbGP1 was characterized by a series of analytical techniques including methylation analysis, partial acid hydrolysis, GC, GC–MS and ESI-MS. These analysis results were first reported in *L. barbarum* which could provide a foundation for the further investigation on the structure–activity relationship and healthy food industry application of *Lycium* polysaccharides.

### 2. Material and methods

#### 2.1. Materials and chemicals

The dried mature fruits of *L. barbarum* were the product of Ning Xia Huizu Autonomous Region, People's Republic of China. All standard monosaccharides (D-galactose, l-rhamnose, l-arabinose, D-glucose, D-xylene, D-fucose and D-mannose), dextrans (5000, 12,000, 25,000, 50,000, 80,000 and 150,000 Da) and Dowex 50 WX-8–400 cation exchange resin were purchased from Sigma (St. Louis, MO, USA). Sephadex G-100 was purchased from Pharmacia. DEAE-Cellulose S2 was purchased from Hengxin Chemical Reagent Co. (Shanghai, China). All other chemicals used were of analytical grade.

#### 2.2. Purification of LbGP1

The crude polysaccharide LBP was obtained from the fruit of *L. barbarum* using water extraction followed by ethanol precipitation as described previously (Huang et al., 1998). The LBPI was further purified with gel permeation chromatography on a Sephadex G-100 column (1.5 cm × 120 cm) using 0.1 mol/L NaCl solution as an eluent. Each fraction was collected and monitored by UV absorption at 280 nm for protein content and at 490 nm for total carbohydrate content using a phenol–H₂SO₄ assay. The fractions designed as the main carbohydrate-containing peaks of the chromatography elution profile were pooled and concentrated. After dialysis and lyophilization, a white fluffy polysaccharide LbGP1 was obtained.

#### 2.3. Determination of the homogeneity and the molecular weight

The homogeneity and molecular weight of LbGP1 were evaluated and determined by high performance gel permeation chromatography (HPGPC). HPLC was performed on a Waters 2695 HPLC system coupled with TSK-Gel G4000SW column and Waters 2414 RI detector. The determination procedure was carried out according to the methods described previously by Lv, Wang, Cheng, Huang, and Wang (2013).

#### 2.4. Determination of sugar and protein contents

The carbohydrate content was determined by the phenol–H₂SO₄ method using glucose as a standard (Lin, Wu, Wu, & Tian, 1996). The protein content was estimated by the Bradford’s method using ovalbumin as a standard (Bradford, 1976).

#### 2.5. Sugar composition analysis by GC

The sample was hydrolyzed under 2 mol/L trifluoroacetic acid (TFA) at 120 °C for 2 h, and then evaporated to dryness to remove the acid. The dried hydrolyzed sample was dissolved and incubated in 250 μL 1 M ammonium hydroxide containing 10 mg/mL NaBH₄ at room temperature for 3 h. After the borate ions removed completely, the sample was acetylated with 0.2 mL pyridine and 0.2 mL acetic anhydride overnight at room temperature and then dried under an N₂ stream. A mixture of chloroform and water was added to the sample, followed by vortexing. The organic phase was concentrated under a N₂ stream and analyzed by GC. GC analysis was conducted on Shimadzu GC2010 with Rtx-50 capillary column (0.25 mm × 30 m) and the temperature program was: 180 °C for 2 min, then to 210 °C at 6 °C/min, then increasing to 215 °C at 0.3 °C/min, finally reached 240 °C at 6 °C/min. N₂ was used as the carrier gas at 0.6 mL/min.

#### 2.6. Carbohydrate–peptide linkage analysis

The carbohydrate–peptide linkage of LbGP1 was analyzed by the β-elimination reaction. LbGP1 (10 mg/mL) was incubated in 0.1 mol/L NaOH containing 1.0 mol/L NaBH₄ at 45 °C for 24 h, and then the sample was scanned from 200 nm to 400 nm by UV spectrophotometer. The obtained data was compared with that of the alkali untreated sample.

#### 2.7. Release of the glycans from LbGP1

80 mg of LbGP1 were dissolved in 3.5 mL reaction buffer (100 mM Tris–HCl, pH 8.0, 1 mM CaCl₂). Then 0.8 mg Pronase E were added into the solution and incubated at 37 °C for 72 h, with further amounts of 0.8 mg Pronase E was added for every 24 h. The resulting product was isolated on Sephadex G-100 column, eluted with 0.1 mol/L NaCl at a flow rate of 0.5 mL/min, and monitored by UV absorption at 280 nm and at 490 nm using phenol–H₂SO₄ assay. The fractions containing sugar were collected, dialyzed against distilled water and then lyophilized, designed as LbGP1-OL.

#### 2.8. Methylation analysis

The samples were fully methylated using modified Ciucanu method as described previously (Needs & Swivendran, 1993) and then hydrolyzed, reduced, and peracetylated according to the
The partially methyalted alditol acetates were analyzed on a Shimadzu GCMS-QP2010 system equipped with a capillary column of rtx-5 ms (30.0 m × 0.25 mm × 0.25 μm), and the temperature program was: 130 °C (or 140 °C) for 40 min, then to 250 °C at a rate of 2 °C/min. Helium was used as the carrier gas and maintained at 1.24 mL/min.

2.9. Partial acid hydrolysis

LbGp1-OL was hydrolyzed under 20 mmol/L H2SO4 at 80 °C for 12 h. The solution was dialyzed against distilled water for 72 h using a dialysis bag with a MWCO of 8 KDa. The non-dialyzable (inside the dialysis bag) sample was concentrated and lyophilized, giving the partially acid hydrolyzed polysaccharide LbGp1-OL-P. The dialyzable (outside the dialysis bag) fraction was concentrated and neutralized with barium carbonate. After centrifugation, the supernatant was freeze-dried and designated as LbGp1-OL-O.

2.10. Electrospray ionization mass spectrometer (ESI-MS) analysis

The sample was desalted using DOWEX 50 WX 8–400 cation-exchange column according to our previously described method (Wang, Fan, Zhang, Wang, & Huang, 2011) and then dissolved in methanol for the ESI-MS analysis. ESI-MS analysis was carried out on Thermo Scientific LTQ XL ion trap mass spectrometer (USA) in the positive ion mode. Samples were injected into the ion source by a 2 μL fixed quantity (Rheodyne) loop, and the mobile phase was methanol/water (1:1, v/v) solution at a flow rate of about 200 μL/min. The MS analysis conditions were adopted according to those we recently described (Wang et al., 2011).

3. Results

3.1. Purification of LbGp1

The brown crude polysaccharide (0.42% yield of the crude herb), designed as LBP, was obtained from the fruit of L. barbarum L. by a series of processing steps, including water extraction, ethanol precipitation, deproteinization, dialysis and lyophilization. LBP was purified by anion exchange chromatography, yielding five polysaccharide subfractions LPB1, LPB2, LPB3, LPB4 and LPB5. LPB1 was further purified by gel permeation chromatography on a Sephadex G–100 column (Fig. S1). A water-soluble, white and fluffy polysaccharide, termed as LbGp1, was obtained with a yield of 0.018% of the crude herb after collection (15–24 tubes), dialysis and lyophilization.

3.2. Homogeneity and molecular weight

The homogeneity and molecular weight of LbGp1 were determined by HPGPC. LbGp1 eluted as a single and symmetrical peak from gel-permeation chromatography on TSK G4000SW column, indicated that LbGp1 was homogeneous glycoconjugate (data was not shown). The average molecular weight (MW) of the sample was estimated to be 49.1 KDa based on the calibration with standard dextrans.

3.3. Monosaccharide composition analysis of LbGp1

The sugar composition of LbGp1 determined by GC was shown in Fig. S2. LbGp1 consisted of only Ara and Gal in the molar ratio of 5.61. Results from colorimetric assay showed that LbGp1 contained 95.65% carbohydrate and 3.75% protein contents. A UV scan in the region of 200–400 nm showed strong absorbance at about 200 nm but weak absorbance at 280 nm, indicating further that the LbGp1 sample contained a trace of protein.

In the FT-IR spectrum of LbGp1 (Fig. S3), The major broad absorptions in the region of 3600–3200 cm⁻¹ and the small band at around 2932.51 were due to the O–H and C–H stretching and bending vibrations, respectively; the relatively strong absorption peak at 1647.19 cm⁻¹ reflected the absorption of the C=O group that is part of glycosides. The strong absorptions in the range of 950–1160 cm⁻¹ indicated a pyranose form of sugars (Zhao, Kan, Li, & Chen, 2005). While the weak absorbance at 905 cm⁻¹ suggested the existence of pyranoses in the β-configuration (Zhang, 1994). The IR spectrum showed no specific signature of uronic acid.

3.4. Glycosidic linkage analysis

There are two major types of protein glycosylation: N-glycosylation and O-glycosylation. Manose (Man) and N-acetylglucosamine (GlcNAc) were required for N-linked glycosylation. Sugar compositional analysis showed no Man and GlcNAc in LbGp1, the carbohydrate-peptide linkage of LbGp1 was predicted to be O-linkage type. To further validate carbohydrate–peptide linkage of LbGp1, the β-elimination method was employed to analyze the O-linked glycan of LbGp1. O-linkage glycan was released under non-reducing weak alkali conditions, serine and threonine were turned into α-aminoacrylic and α-amino crotonic acid which would enhanced absorbance at 240 nm. Compared with the native LbGp1, the alkali-treated LbGp1 sample had remarkable absorbance at 240 nm (Fig. 1), showing that the β-elimination reaction had taken place. The data demonstrated that the linkage between the glycan and the core protein backbone was O-linkage in LbGp1 (Lee, Cho, Song, Hong, & Yoo, 1996).

3.5. Methylation analysis of LbGp1-OL

After being released by Pronase E digestion, the carbohydrate fraction of LbGp1 was purified on Sephadex G-100 column (data was not shown), affording a product, LbGp1-OL.

To determine the substitution pattern of the monosaccharide in the glycan, the fully methylated LbGp1-OL was hydrolyzed with acid to produce alditol acetates and analyzed by GC–MS. Its total ion chromatogram (TIC) profile was shown in Fig. 2. Peaks of methylated sugars were identified by their retention times and mass spectra.

The peak area ratios of the fragments were used as relative molar ratio, but molar response factors of partially methylated alditol acetates were calculated by the ‘effective carbon response’, according to the predicted ionization potential of organic constituents in a Flame Ionization Detector (Sweet, Shapiro, & Albersheim, 1975). The GC–MS results were shown in Table 1. Important conclusions can be conducted from Table 1: (1) The amount of Ara in partially methylated alditol acetate mixtures of LbGp1 accounted for about 85% of the total carbohydrate which is consistent with the results of its sugar composition. (2) The Ara residues were fucosidase: the Gal residues were pyranoside: (3) the non-reductive terminals were Ara (1→ only; (4) the branching residue were →3, 6) Gal (1→, which took half part of all hexoses. Thus, LbGp1-OL was proposed to be a highly branching polysaccharide. However, we could not affirm whether the backbone of LbGp1-OL was composed of →3) Gal (1→ or →6) Gal (1→ residues.

3.6. Partial acid hydrolysis

Furanose is easily hydrolyzed in diluted H2SO4 with a rate of nearly 2 orders of magnitude higher than pyranose (Wu, 1987). However, furanose (arabinose) was abundant in LbGp1-OL. So...
the partial hydrolysis was carried out to determine the structure of the main chain.

The dialyzable and non-dialyzable fractions were lyophilized and designated as LbGp1-OL-O and LbGp1-OL-P, respectively. After sugar composition analysis, the results showed that LbGp1-O was composed of Ara only, and LbGp1-OL-P was composed of Ara and Gal with a relative molar ratio of 1.0: 12.5. LbGp1-OL-P was fully methylated and their partially methylated alditol acetates were analyzed by GC and GC–MS (Fig. 3). The comparison of methylation results of LbGp1-OL and LbGp1-OL-P were shown in Table 2.

![Fig. 1. UV spectrum profiles of the native LbGp1 and the alkali-treated LbGp1 sample.](image)

![Fig. 2. TIC chromatogram of partially methylated alditol acetates of LbGp1-OL.](image)

![Fig. 3. TIC chromatogram of partially methylated alditol acetates of LbGp1-OL-P.](image)

**Table 1**

<table>
<thead>
<tr>
<th>Peak No.</th>
<th>Methylated sugar</th>
<th>Linkages types</th>
<th>Relative molar ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2,3,5-Me3-Ara</td>
<td>Ara[1→</td>
<td>4</td>
</tr>
<tr>
<td>2</td>
<td>3,5-Me2-Ara</td>
<td>→2</td>
<td>Ara[1→</td>
</tr>
<tr>
<td>3</td>
<td>2,5-Me2-Ara</td>
<td>→3</td>
<td>Ara[1→</td>
</tr>
<tr>
<td>4</td>
<td>2,3,6-Me3-Gal</td>
<td>→3,Galp[1→</td>
<td>2</td>
</tr>
<tr>
<td>5</td>
<td>2,4,6-Me2-Gal</td>
<td>→3,Galp[1→</td>
<td>2</td>
</tr>
<tr>
<td>6</td>
<td>2,4-Me2-Gal</td>
<td>→3,6,Galp[1→</td>
<td>4</td>
</tr>
</tbody>
</table>

* 2,3,5-Me3-Ara = 1,4-di-O-acetyl-2,3,5-tri-O-methyl-arabinose, etc.

**Table 2**

<table>
<thead>
<tr>
<th>Methylated sugar</th>
<th>Linkages types</th>
<th>LbGp1-OL</th>
<th>LbGp1-OL-P</th>
</tr>
</thead>
<tbody>
<tr>
<td>2,3,5-Me3-Ara</td>
<td>Ara[1→</td>
<td>8</td>
<td>–</td>
</tr>
<tr>
<td>3,5-Me2-Ara</td>
<td>→2</td>
<td>Ara[1→</td>
<td>54</td>
</tr>
<tr>
<td>2,5-Me2-Ara</td>
<td>→3</td>
<td>Ara[1→</td>
<td>28</td>
</tr>
<tr>
<td>2,3,4,6-Me2-Gal</td>
<td>Galp[1→</td>
<td>4</td>
<td>–</td>
</tr>
<tr>
<td>2,4,6-Me2-Gal</td>
<td>→3,Galp[1→</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td>2,3,4-Me2-Gal</td>
<td>→6,Galp[1→</td>
<td>–</td>
<td>5</td>
</tr>
<tr>
<td>2,4-Me2-Gal</td>
<td>→3,6,Galp[1→</td>
<td>8</td>
<td>3</td>
</tr>
</tbody>
</table>

* 2,3,5-Me3-Ara = 1,4-di-O-acetyl-2,3,5-tri-O-methyl-arabinose, etc.

The dialyzable and non-dialyzable fractions were lyophilized and designated as LbGp1-OL-O and LbGp1-OL-P, respectively. After sugar composition analysis, the results showed that LbGp1-O was composed of Ara only, and LbGp1-OL-P was composed of Ara and Gal with a relative molar ratio of 1.0: 12.5. LbGp1-OL-P was fully methylated and their partially methylated alditol acetates were analyzed by GC and GC–MS (Fig. 3). The comparison of methylation results of LbGp1-OL and LbGp1-OL-P were shown in Table 2.
As was shown in Table 2, LbGp1-OL-P had no terminal Arae, (1 → 2)-linked Aræ and (1 → 3)-linked Aræ, indicating that they may exist as branches. The decrease of five → 3,6Galp(1→ branch- ing unit in LbGp1-OL-P was concomitant with the increase of five →6Galp(1→ in LbGp1-OL-P, suggesting that the backbone of the LbGp1-OL was composed of →6Galp(1→, with Gal residues substituted at O-3 of arabinosyl groups. In addition, the three new terminal Galp(1→ in LbGp1-OL-P were equal to the decrease of →3Galp(1→ in LbGp1-OL, indicating (1→ 3)-linked Galp in LbGp1-OL was located at branches and were substituted by arabinosyl groups at O-3 site. It can be deduced from the results that the backbone of LbGp1-OL was composed of (1→ 6)-linked Galp.

3.7. ESI-MS analysis

After desalted using DOWEX 50 WX 8–400 cation-exchange column, LbG1-OL-O was analyzed on a Thermo LTQ XL mass spectrometer. The MS profiling of it was shown in Fig. S4. All the molecular ions refer to [M+Na+] adducts at m/z 305.02, 437.05, 569.17, 701.20, 833.15, 965.24 and 1097.13, can be assigned to the molecular ions of arabinose oligomers with degree of polymerization (DP) from 2 to 8. The result suggested that the side chain attached to LbGp1-OL was composed of Ara with a variety of DP, in consistent with the sugar composition and methylation analysis.

Based on the above results, we suggested the structure of the repeating unit of LbGp1-OL could be represented as follows (Fig. 4).

![Fig. 4. The hypothetical structure of the repeating unit of LbGp1-OL.](http://dx.doi.org/10.1016/j.foodchem.2014.02.171)

4. Conclusion

Based on monosaccharide composition, partial hydrolysis, methylation analysis and electrospray ionization mass spectrometer (ESI-MS), LbGp1 was identified to be a highly branched polysaccharide with a backbone of →6Galp(1→ linked galactose substituted at O-3 by galactosyl or arabinosyl groups. This structure belongs to arabinogalactan (Clarke, Anderson, & Stone, 1979), which is composed of Ara and Gal, and sometimes links to protein by covalent bond. The arabinogalactan was commonly found in plant, which possessed a series of functions, such as immuno-regulation, stimulating NK cell, and drug delivery (Pennell, Janniche, & Kjellbom, 1991; Pennell, Knox, & Scofield, 1989; Pennell & Roberts, 1990). Therefore, the potential biological activates of LbGp1 is worth to be further explored.

Acknowledgements

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.foodchem.2014.02.171.

References


