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Note

# Structural characterization of the glycan part of glycoconjugate LbGp2 from *Lycium barbarum L.*

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**Abstract**

A glycoconjugate with pronounced immunoactivity, designated as LbGp2, was isolated from the fruit of *Lycium barbarum L.* and purified to homogeneity by gel-filtration. Its carbohydrate content is up to 90.71% composed of Ara, Gal and amino acids. The molecular weight is 68.2 kDa as determined by size exclusive chromatography (SEC). The complete structure of the repeat unit of the glycan of LbGp2 was elucidated based on glycosidic linkage analysis, total acid hydrolysis, partial acid hydrolysis, $^{1}H$ and $^{13}C$ NMR spectroscopy. According to the experiments, the glycan possesses a backbone consisting of (1→6)-β-galactosyl residues, about fifty percent of which are substituted at C-3 by galactosyl or arabinosyl groups and the major nonreducing end being made of Ara (1→. 

*Keywords:* Structural characterization; Structure; Repeating unit; *Lycium barbarum L.*

Since the late 1980s, arabinogalactan-proteins (AGPs) from plants have attracted widespread attention. They are localized in cytoplasmic organelles,[1,2] plasma membranes,[3,4] cell walls,[5] and extracellularly in the medium of suspension-cultured cells.[4,6] AGPs are thought to play important roles in cell differentiation,[7,8] development,[9,10] and cell–cell interactions, for elongation growth of the cell wall,[11] and for defence systems in plant.[12]

*Lycium barbarum L.*,[13] is a famous traditional Chinese herbal medicine which has functions of 'nourishing the kidney and producing essence, nourishing the liver and brightening eyes'. It has been widely used as health-giving food for 2300 years. Until now five glycoconjugates LbGp1–LbGp5 have been isolated from *L. barbarum L.* and three of them have been elucidated.[14–16] We found that the extractable glycoconjugates show high immunoactivity. In this paper, the complete structural analysis of the glycan part of LbGp2 is reported.

## 1. Results and discussion

Lbp2 was isolated from *L. barbarum L.* as described previously.[13] After being further purified on a Sephadex G-100 column, a homogeneous glycoconjugate named LbGp2 was obtained. Chemical homogeneity of LbGp2 was checked by HPLC and CE. The molecular weight was 68.2 kDa, determined by Sepharose 4B column, using T-dextran as standard.

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After being released by β-elimination, the glycan of LbGp2 was purified by Sephadex G-100 column. The saccharide fraction was collected and named LbGp2-OL. Elemental analysis showed C 41.87%, H 6.02% and N 0%. Monosaccharide composition determined by gas chromatography gave Ara and Gal in a molar ratio of 4:5.

To determine the linkage of monosaccharides in the glycan, LbGp2-OL was subjected to a GC–MS linkage analysis (Table 1).

The peak area ratio of methylated fragments was used as a molar ratio, but molar response factors of partially methylated alditol acetates are calculated by the 'effective carbon response', originally based on the predicted ionization potential of organic constituents in an FID.[17] From Table 1, important structural conclusions could be drawn: (1) The major nonreducing end was Ara(1→ instead of Gal(1→ and half of Ara were terminal. (2) The branching unit was →3,6)Gal(1→, which was about 60% of all hexoses. Thus the glycan was highly branched. As to the glycosyl ring forms, it could be deduced that peaks 1 and 2 were furanoses and peaks 4, 5, 6, and 7 were pyranoses. However, peak 3 could either be 1,4-linked arabinopyranose or be 1,5-linked arabinofuranose. In addition, we could not know whether the backbone was made of →3)Gal(1→ or →6)Gal(1→. Partial acid hydrolysis was carried out for the abundant arabinoses, because furanose is easily hydrolyzed in dilute sulphuric acid with a rate of nearly two orders of magnitude higher than pyranose.[18] Thus LbGp2-OL was hydrolyzed in dilute sulphuric acid, monitored by paper chromatography. The hydrolysate was named LbGp2-OL′. Gas chromatography showed that LbGp2-OL′ was composed of Gal only. Results of glycosidic linkage analysis of LbGp2-OL′ were compared with those of LbGp2-OL. As shown in Table 2, the increase of six →6)Gal(1→ in LbGp2-OL′ was concomitant with the decrease of seven →3,6)Gal(1→ branching unit in LbGp2-OL, suggesting (1) the main chain of LbGp2-OL′ was composed of →6)Gal(1→. (2) Seven →3,6)Gal(1→ were substituted at C-3 by arabinosyl residue. (3) One of →6)Gal(1→ was substituted at C-6 by arabinosyl residue. In addition, the three new terminal Gal(1→ in LbGp2-OL′ was accompanied by the decrease of two →3)Gal(1→ in LbGp2-OL, implied that two →3)Gal(1→ were substituted by Ara(1→.

To determine the anomeric configuration, methylglycosides were selected as model compounds as shown in Table 3.[19,20]

$^{1}H$ and $^{13}C$ NMR spectra of LbGp2-OL were compared with those of LbGp2-OL′ and the deduced data were shown in Table 4.

As shown in Table 4, $^{1}H$ 5.30 ppm and $^{13}C$ 110–109 ppm in LbGp2-OL were not found in LbGp2-OL′, so Ara(1→ was α-furanose. $^{1}H$ 5.30 ppm and 5.18–5.15 ppm in LbGp2-OL disappeared in LbGp2-OL′ and the ratio of integral area between $^{1}H$ 5.30 ppm and 5.18–5.15 ppm was 1.3:1, therefore, →3)Ara(1→ and →5)Ara(1→ were β-furanoses. From Table 3, it could be deduced that all Gal in glycan were β-pyranoses which was correspondent to the characteristic absorption at 890 $cm^{-1}$ in IR spectrum. Therefore, it was concluded that the structure of the repeating unit of LbGp2-OL was as shown in Fig. 1.

Fig. 1 is only one of the possible structures and the branches can be arranged in other

Table 1
GC–MS data for LbGp2-OL

| Peak | Fragment | Configure | Mole (%) | No. of residues per repeating unit |
|---|---|---|---|---|
| 1 | 2,3,5-Me3-Ara | Ara(1→ | 9.74 | 10 |
| 2 | 2,5-Me2-Ara | →3)Ara(1→ | 2.21 | 2 |
| 3 | 2,3-Me2-Ara | →4 or 5)Ara(1→ | 4.23 | 4 |
| 4 | 2,3,4,6-Me4-Gal | Gal(1→ | 0.985 | 1 |
| 5 | 2,4,6-Me3-Gal | →3)Gal(1→ | 4.75 | 5 |
| 6 | 2,3,4-Me2-Gal | →6)Gal(1→ | 2.83 | 3 |
| 7 | 2,4-Me2-Gal | →3,6)Gal(1→ | 10.95 | 11 |

Table 2
Glycosidic linkage analysis of LbGp2-OL and LbGp2-OL′

| GC peak | Configure | LbGp2-OL | | LbGp2-OL′ | |
|---|---|---|---|---|---|
| | | Mole (%) | No. of residues per repeating unit | Mole (%) | No. of residues per repeating unit |
| 1 | Ara(1→ | 9.47 | 10 | | |
| 2 | →3)Ara(1→ | 2.21 | 2 | | |
| 3 | →4 or 5)Ara(1→ | 4.25 | 4 | | |
| 4 | Gal(1→ | 0.985 | 1 | 4.04 | 4 |
| 5 | →3)Gal(1→ | 4.75 | 5 | 2.81 | 3 |
| 6 | →6)Gal(1→ | 2.83 | 3 | 9.02 | 9 |
| 7 | →3,6)Gal(1→ | 10.95 | 11 | 4.22 | 4 |

reasonable orders. This kind of structure is found commonly in plant arabinogalactan-proteins. In general, the carbohydrate moiety of these arabinogalactan-proteins comprises arabino-3,6-galactan (type II) and the protein moiety is characteristically rich in threonine, serine, alanine and hydroxyproline.[21] While sharing general structural similarity, LbGp2 was distinguishable by its immunoactivity. In our experiments, the glycocojugates in *L. barbarum L.* had direct elevation on splenocyte proliferation in mice as measured using $^{3}$H TdR incorporation assay and the effects of glycan chains were stronger than that of respective glycoconjugates.[22] The results suggested that the immunoactive components of the fruit of *L. barbarum L.* were a kind of glycan with complex structure. In addition, glycoconjugate could increase the expression of IgG of mice spleen lymphocyte, and inhibit the growth of 7721 cell. They were different in inhibiting the LDL peroxidation[23] and cleaning away oxygen free radical.

## 2. Experimental

*Material.*—*L. barbarum L.* was the product of Ning Xia Huizu Autonomous Region, People's Republic of China. Sephadex G-100 and CM-Sephadex C-50 were purchased from Ammersham Pharmacia Biotech. T-dextran series of different standard molecular weights were from Fluka.

*General.*—Concentrations were performed under diminished pressure at a bath temperature not exceeding 45 °C. The spectrophotometer 722 for colorimetric analysis was the product of Shanghai Third Analytical Instrument Factory. The infrared spectrum (IR) of the methylated polysaccharide was recorded on a Bio-Rad FTS 185 spectrometer. High-performance liquid-chromatography (HPLC) was performed on Shimadzu LC-10AD equipped with BIO-SEP-S4000 exclusion column and water as solvent (0.4 mL/min); the elute was monitored by RI detectors. Capillary electrophoresis (CE) was performed on Water Quanta 4000 E using 0.1 mol/L boric

Table 3
Anomeric $^{1}$H and $^{13}$C NMR chemical shifts of some methyl-glycosides

| | $^{1}$H (ppm) | | $^{13}$C (ppm) | |
|---|---|---|---|---|
| | p | f | p | f |
| α-D-Ara | 4.16 | 5.28 | 105.1 | 109.2 |
| β-D-Ara | 4.72 | 5.12 | 102.5 | 103.2 |
| α-D-Gal | 4.73 | | 99.2 | 103.5 |
| β-D-Gal | 4.20 | | 104.5 | 109.6 |

Table 4
Anomeric $^{1}$H and $^{13}$C NMR chemical shifts of LbGp2-OL and LbGp2-OL′

| | LbGp2-OL (ppm) | LbGp2-OL′ (ppm) | |
|---|---|---|---|
| $^{1}$H NMR | 5.30 | | α-Ara$_f$ |
| | 5.18 | | β-Ara$_f$ |
| | 5.15 | | |
| $^{13}$C NMR | 111–110 | | α-Ara$_f$ |
| | 105–106 | 106 | β-Gal$_p$ |
| | 103–104 | | β-Ara$_f$ |

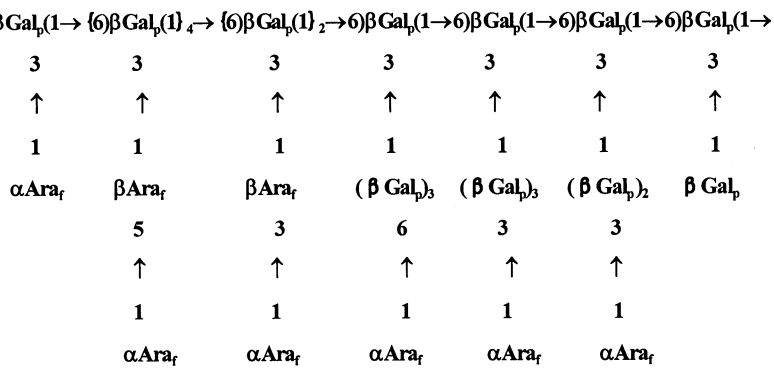


Fig. 1. The complete structure of the repeat unit of the glycan of LbGp2.

acid–NaOH buffer (pH 10) as solvent, detected at 20 V and 254 nm. Gas chromatography of the alditol acetate derivative was conducted on Varian VISTA402 equipped with 3% OV-225 capillary column (0.3 mm × 25 m). The GC oven was held at 65 °C for 1 min before being increased to 290 °C at a rate of 8 °C/min. Paper chromatography (PC) was performed on Whatman No. 1 paper in the following solvent system: 3:2:1 *n*-butanol–pyridine–water, the sugars being detected with aniline–phthalic acid. GC–MS analysis of partially methylated alditol acetates was effected on Shimadzu QP 5000 equipped with OV-17 capillary column (0.2 mm × 30 m), using a temperature gradient of 140–200 °C at 5 °C/min, increased to 300 °C at 10 °C/min. NMR spectrum was obtained on a Bruker-MX-300 spectrometer equipped with a dual probe, in the FT mode at rt.

*Purification of LbP2.*—LbP2 was isolated from *L. barbarum L.* as described previously.[13] Lbp2 was further purified on Sephadex G-100 column (0.75 × 115 cm) eluted with 0.1 N NaCl at a flow rate of 0.8 ml/L, monitored by UV absorption at 280 nm and phenol–$H_2SO_4$ assay at 490 nm. The eluate was separated into two parts by collecting fraction nos. 13–20 (**1**), 13–20 (**2**) (Fig. 2). Sub-fraction **1** was further purified using CM-Sephadex C-50 to obtain a glycoconjugate named LbGp2. The homogeneity of LbGp2 was detected by HPLC and CE.

*Determination of the molecular weight ($M_w$).*—LbGp2 was applied to a Sepharose 4B (2 × 50 cm) column monitored by phenol–$H_2SO_4$ method, eluted with 0.1 M KCl at a flow rate of 0.5 mL/min. The column was calibrated using T-dextran series of different molecular weights.

*Bata-elimination of LbGp2.*—LbGp2 was hydrolyzed in 0.1 M NaOH–1.0 M $NaBH_4$ at 45 °C for 72 h and then neutralized with 2.0 M $CH_3COOH$. After concentration, the hydrolysate was purified on a Sephadex G-100 column, eluted with 0.1 M NaCl at a flow rate of 0.5 mL/min. Eluted fractions were monitored by UV absorption at 280 nm, and by phenol–$H_2SO_4$, absorption at 490 nm. The fractions containing sugar were collected, then dialyzed against distilled water and then lyophilized.

*Monosaccharide composition of LbGp2-OL.*—The sample was hydrolyzed in 1.0 M $H_2SO_4$ at 100 °C for 4 h, neutralized with barium carbonate, reduced to alditol by $NaBH_4$ with trace ammonia solution (25%), and acetylated with 1:1 $Ac_2O$–pyridine (v/v) at rt overnight.[24] The alditol acetate derivative after hydrolysis was analyzed by GC.

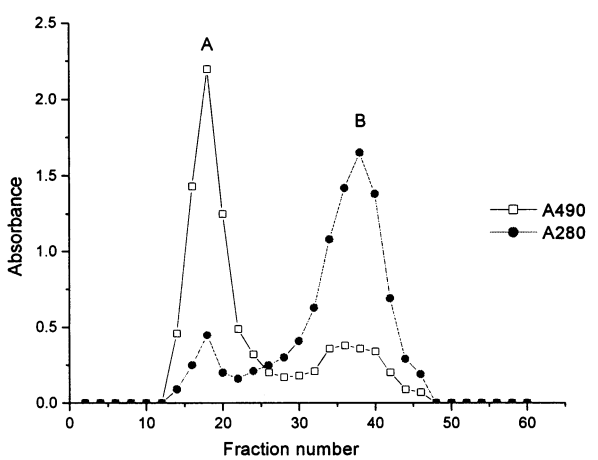


Fig. 2. Lbp2 was purified on Sephadex G-100 column (0.75 × 115 cm) eluted with 0.1 N NaCl at a flow rate of 0.5 ml/L, 7 min/tube.

*Methylation analysis.*—The sample was methylated with DMSO–SMSM–$CH_3I$ containing $Me_4U$,[25] then methylated again with NaOH (solid)–$CH_3I$–DMSO,[26] hydrolyzed in HCOOH (88%) at 100 °C for 3 h, and 0.125 M $H_2SO_4$ at 100 °C for 16 h, then transferred into alditol acetates as before. GC–MS measurement of the partially methylated alditol acetates was carried out on Shimadzu QP 5000.

*Partial acid hydrolysis.*—The sample was hydrolyzed with 20 mM $H_2SO_4$ at 80 °C for 12 h. The process of hydrolysis was monitored by paper chromatography. The hydrolysate was dialyzed against distilled water and then lyophilized.

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