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Isolation and structural characterization of the polysaccharide LRGP1 from *Lycium ruthenicum*

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ABSTRACT

A novel water-soluble glycoconjugate, designated as *Lycium ruthenicum* glycoconjugate polysaccharide 1 (LRGP1), was isolated from the fruits of *Lycium ruthenicum* Murr. The crude polysaccharide was obtained by hot water extraction and purified by ion-exchange and gelfiltration chromatography. Its molecular weight was 56.2 kDa determined by HPGPC (high performance gel permeation chromatography). Monosaccharide composition analysis revealed that it was composed of rhamnose, arabinose, xylose, mannose, glucose, and galactose in a molar ratio of 0.65:10.71:0.33:0.67:1:10.41. The existence of O-type carbohydrate–peptide linkage in LRGP1 was demonstrated by β-elimination reaction. On the basis of monosaccharide composition, partial acid hydrolysis, methylation analysis and ESI-MS analysis, LRGP1 was characterized as a branched polysaccharide rich in arabinose and galactose with a backbone composed of (1 → 3)-linked Gal. The branches were composed of (1 → 5)-linked Ara, (1 → 2)-linked Ara, (1 → 6)-linked Gal, (1 → 3)-linked Gal, (1 → 4)-linked Gal and (1 → 2,4)-linked Rha. Arabinose, xylose, mannose, and glucose were located at the terminal of the branches.

1. Introduction

Lycium ruthenicum Murr. belongs to the Solanaceae family and which is widely distributed in the salinized desert of Qinghai-Tibet Plateau. Its special physiological characteristics of drought-resistance and salt-resistance make it an ideal plant for preventing soil desertification and alleviating the degree of soil salinity–alkalinity. In addition, *L. ruthenicum* is a famous traditional Chinese herb which has been used for treatment of heart disease, irregular menstruation and menopause. *L. ruthenicum* contains many functional components such as pigments, essential oils and polysaccharides. The antioxidant activity and ability to enhance immunity of pigments have been demonstrated (Li, Qu, Zhang, & Lv, 2006), and the composition of pigments and essential oils in *L. ruthenicum* has been well documented (Altintas, Kosar, Kirimer, Baser, & Demirci, 2006; Zheng et al., 2011).

Polysaccharides exist in many Chinese herbs, and modern pharmacological studies have shown that herbal polysaccharides possess antioxidant, hypoglycemic, anti-fatigue, anti-tumor and other biological activities, which are hotspots of the research on functional factors of drugs (Garcia-Gonzalez, Alnaief, & Smirnova, 2011; Rodriguez-Gonzalez et al., 2011; Xiong, Li, Huang, Lu, & Hou, 2011; Yapo & Koffi, 2008). Crude polysaccharides isolated from *L. ruthenicum* were shown to have hypoglycemic and anti-fatigue effects (Feng, He, & Chen, 2009; Wang, Chen, & Zhang, 2009). Up to now most of the researches were focused on the extraction method and bioactivities, no comprehensive studies have been conducted to explore the structural characteristics of *L. ruthenicum* polysaccharides. This has restricted the further research and application of *L. ruthenicum*.

Both *L. barbarum* and *L. ruthenicum* belong to the Solanaceae family. Numerous studies have demonstrated that the bioactive components of *L. barbarum* fruit is attributed mainly to its polysaccharide–protein complex (LBGP). It was reported that LBGP was an arabinogalactanprotein containing (1 → 4)-linked Gal backbone (Huang, Lin, Tian, & Ji, 1998) and could enhance the immune function, protect liver from damage, lower blood glucose level, reduce the side effects of chemotherapy and radiotherapy (Ke et al., 2011; Liang, Jin, & Liu, 2011). Therefore, it is important to study the structure and bioactivity of *L. barbarum* polysaccharides.

Abbreviations: CLRP, crude *Lycium ruthenicum* polysaccharides; LRGP1, *Lycium ruthenicum* glycoconjugate polysaccharide 1; LRGP1-OL, the glycan of the LRGP1; HPGPC, high performance gel permeation chromatography.

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In this study, we reported a novel water-soluble polysaccharide extracted from the fruit of *L. ruthenicum*. Its isolation, homogeneity, molecular weight, monosaccharide composition, and structural characteristics were investigated. All of these results were first reported in *L. ruthenicum* polysaccharide, and these results provided a basis for future pharmacological studies.

2. Materials and methods

2.1. Plant material

The dried mature fruit of *L. ruthenicum* was purchased from Jiahe Biological Engineering Co. (Qinghai, China). It was dried at 50 °C and crushed into powder.

2.2. Chemicals

Standard monosaccharides (arabinose, rhamnose, fucose, xylose, galactose, glucose, mannose, glucuronic acid and galacturonic acid) and glucose oligomers were purchased from Sigma Chemical Co. (USA); DEAE-Cellulose-52 was purchased from HengXin Chemical Reagent Co. (Shanghai, China); Sephadex G-100 was purchased from Pharmacia Co. (Uppsala, Sweden); other reagents used were of analytical grade.

2.3. Analytical methods

The carbohydrate content was determined by the PhOH–H_2SO_4 method using glucose as a standard (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956). The protein content was estimated by the method of Bradford using ovalbumin as a standard (Bradford, 1976). All the measurements were repeated three times. A Lambda 25 UV/VIS spectrophotometer was used to detect the absorbance. A UV scan in the region of 200–400 nm was performed on the spectrophotometer. The IR spectrum was determined using an EQUINOX 55 Fourier transform infrared spectrophotometer.

2.4. Isolation of L. ruthenicum polysaccharides

The powder (100 g) of *L. ruthenicum* and 400 mL deionized water were added into a 1000 mL flask. After 2 h of extraction at 70 °C in water bath, the supernatant and the sediment were separated by vacuum filtration. The residue was re-extracted by this procedure. All extraction solutions were pooled and concentrated with a rotary evaporator at a temperature below 40 °C. A fourfold volume of EtOH was added to the extracts to precipitate the polysaccharides overnight at 4 °C. The precipitate was pelleted by centrifugation and redissolved in distilled water. The aqueous solution was treated with Sevag reagent to remove proteins, dialyzed against distilled water for 48 h, concentrated under reduced pressure, and finally lyophilized. A black crude polysaccharide, CLRP, was then obtained.

2.5. Decoloration of crude polysaccharides

CLRP was dissolved in distilled water at 40 °C with continuous stirring and the solution was adjusted to pH 8.8 with $NH_3{\cdot}H_2O$. H_2O_2 (30%) was then added drop-wise until the color faded. After stirring for another 4 h, the color turned primrose yellow. The solution was neutralized with 1 mol/L HCl, followed by dialysis against distilled water and lyophilization.

2.6. Purification of LRGP1

Decolored CLRP (900 mg) was dissolved in water and fractionated on a DEAE-Cellulose-52 anion-exchange column (5.0 cm × 40 cm, HCO_3^- form). The column was first washed with distilled water, and then eluted with a step gradient (0.05, 0.10, 0.25, and 0.50 mol/L $NaHCO_3$) at a flow rate of 1 mL/min. The fractions were monitored by UV absorption at 280 nm for protein content and for total carbohydrate by the PhOH–H_2SO_4 method. The fractions containing sugar were collected, dialyzed and lyophilized, and the obtained residues were designated as LRP1, LRP2, LRP3, LRP4 and LRP5, respectively. LRP1 (60 mg) was further purified by gel permeation chromatography on a Sephadex G-100 column (1.5 cm × 100 cm) using 0.1 mol/L NaCl solution as an eluent. The fractions corresponding to the main carbohydrate-containing peak of the chromatography elution profile were pooled. After dialysis and lyophilization, a white fluffy polysaccharide LRGP1 was obtained. This process was repeated until the necessary quantities of LRGP1 for future experiments were obtained.

2.7. Homogeneity and molecular weight determination

The homogeneity and molecular weight of samples were determined by high performance gel permeation chromatography on a TSK-Gel G3000SW column (7.5 mm × 300 mm). A Waters 2695 high performance liquid chromatography system coupled with a Waters Alliance 2414 refractive index detector was used. The temperature of the column and the detector was kept at 30 °C. The sample concentration was 0.2% (w/v), and the injection volume was 20 μL. The eluent was the mixture of 0.1 mol/L phosphate buffer (pH 6.0) and 0.1 mol/L Na_2SO_4, which was passed through Millipore filters (0.45 μm). The flow rate was 0.5 mL/min. The linear regression was calibrated with dextrans 5000, 12,000, 25,000, 80,000, and 150,000. V_t and V_0 were calibrated with glucose and dextran blue (2,000,000), respectively.

2.8. Monosaccharide analysis

The sample (2 mg) was dissolved in 2 mL of 2 mol/L TFA in a screw-capped vial filled with N_2 and hydrolyzed at 120 °C for 2 h. The hydrolyzate was evaporated with a rotary evaporator. Neutral sugars and uronic acids were simultaneously detected by GC using the method described previously (Lehrfeld, 1985). GC was performed by a Shimadzu GC2010 equipped with a capillary column of rtx-5 ms (30.0 m × 0.25 mm × 0.25 μm). The temperature program was: 180 °C for 2 min, then to 210 °C at 6 °C/min, then to 215 °C at 0.3 °C/min, then to 240 °C at 6 °C/min for 45 min. N_2 was used as the carrier gas at 0.6 mL/min.

2.9. Analysis of carbohydrate–peptide linkage

The carbohydrate–peptide linkage of LRGP1 was analyzed by the β-elimination reaction (Zhu & Zhou, 2005). LRGP1 (10 mg/mL) was incubated in 0.2 mol/L NaOH containing 1.0 mol/L $NaBH_4$ 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 sample without alkali treatment.

2.10. Releasing glycans from LRGP1

80 mg of LRGP1 was dissolved in 3.5 mL of 0.1 N Tris–HCl buffer (pH 8.0) containing 0.1% $CaCl_2$. Then, 1% (g/g) Pronase E was added to the sample and incubated at 37 °C for 24 h, followed by another 48 h digestion with a supplement of 0.5% (g/g) Pronase E. During the digestion, 100 μL of PhMe was added to inhibit bacterial growth (Xu, Zhang, Huang, & Wang, 2010). Finally, the glycans LRGP1-OL released from LRGP1 was purified by a Sephadex G-100 column and then lyophilized.

2.11. Partial acid hydrolysis

LRGP1-OL (40 mg) was dissolved in 10 mL of 0.02 mol/L H_2SO_4 and heated at 80 °C for 12 h. After the solution was cooled down to room temperature, the resulting solution was neutralized with NaOH and dialyzed against deionized water using a dialysis bag with a MWCO of 8 kDa, giving rise to dialyzable (outside the dialysis bag) sample and non-dialyzable (inside the dialysis bag) sample. Both dialyzable and non-dialyzable samples were lyophilized. The non-dialyzable fraction was further purified on a Sephadex G-25 column, giving the partially hydrolyzed polysaccharide LRGP1-OL-I; the dialyzable oligomers were designated as LRGP1-OL-O. Sugar compositions of LRGP1-OL-I and LRGP1-OL-O were analyzed by GC. The structure of LRGP1-OL-I was further analyzed by methylation.

2.12. Methylation analysis

Both the samples of LRGP1-OL and LRGP1-OL-I were methylated using modified Ciucanu method as described previously (Needs & Selvendran, 1993). The methylation procedure was repeated three times. Complete methylation was confirmed by the disappearance of O—H absorption band (3700–3100 cm^{-1}) in FTIR spectrum. Next, the samples were hydrolyzed with 2 mol/L TFA (121 °C, 2 h), reduced with $NaBH_4$, and acetylated to convert into their partially methylated alditol acetates. The resulting alditol acetates were analyzed by GC and GC–MS. Peaks of methylated sugars were identified by their mass spectra. Their relative molar ratios were estimated from the peak areas of GC and corresponding response factors. Response factors of partially methylated and alditol acetates are calculated by the effective carbon response (Sweet, Shapiro, & Albersheim, 1975). GC–MS was performed using a Shimadzu instrument GCMS-QP2010 equipped with an electron impact ion source (ionization energy 70 eV). The capillary column used was rtx-5 ms (30 m × 0.25 mm × 0.25 μm); the temperature program was: 140 °C for 2 min, then to 250 °C at 2 °C/min in 30 min. He (Helium) was used as a carrier gas and the flow rate was 0.6 mL/min. The temperatures of the interface and the ion source were 200 °C and 250 °C, respectively.

2.13. ESI-MS of the dialyzable oligosaccharides

1 mg LRGP1-OL-O were dissolved in 0.5 mL of deionized water, and loaded onto a Dowex 50 WX8-400 cation exchange column (1 mL) pre-equilibrated with 3 mL of deionized water. The oligosaccharides were eluted with 3 mL of deionized water, and the eluted fractions were collected and diluted to 1000 times with MeOH for ESI-MS analysis (Wang, Fan, Zhang, Wang, & Huang, 2011). The ESI-MS data were acquired on a Thermo Scientific LTQ XL ion trap mass spectrometer (USA) in positive ion mode. For the electrospray ion source, the spray voltage was set at 4 kV, with a sheath gas (N_2) flow rate of 30 arb, an auxiliary gas (N_2) flow rate of 5 arb, a capillary temperature of 275 °C, a capillary voltage of 350 V, and a tube lens voltage of 250 V. Samples were injected through a Rheodyne loop, a plastic capillary with a volume of 2 μL, and then diluted and taken into the electrospray ion source by a stream of 50% aqueous MeOH at a flow rate of 200 mL/min from the pump of HPLC system to lessen contamination of the instrument. MS data were collected through the Xcalibur software. The scheme for structural characterization of LRGP1 from *L. ruthenicum* was done as set out in Fig. 1.

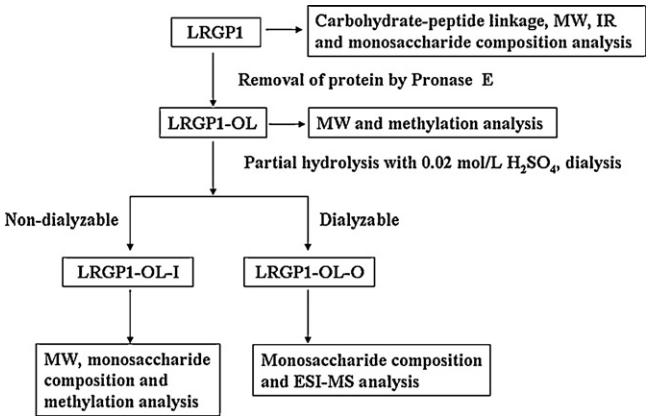

Fig. 1. Scheme for structural characterization of LRGP1 from *Lycium ruthenicum*.

3. Results and discussion

3.1. Isolation and purification of L. ruthenicum polysaccharides

Extraction of the fruit of *L. ruthenicum* with hot water yielded a crude polysaccharide sample, CLRP. The carbohydrate content of CLRP was 66.2% and protein content was 7.3%. CLRP was a black polysaccharide sample in which the pigment could not be removed by column chromatography. To avoid the influence of pigment on the structure analysis, decoloration was performed with 30% H_2O_2. After decoloration, the carbohydrate content of decolored CLRP was 93.2% and protein content was 4.4%. Decolored CLRP was purified by anion exchange chromatography, yielding five polysaccharide subfractions LRP1, LRP2, LRP3, LRP4, and LRP5 (Fig. 2A). All of the five fractions have absorption peaks at A_{490} and A_{280}, indicating that they contained both polysaccharides and proteins.

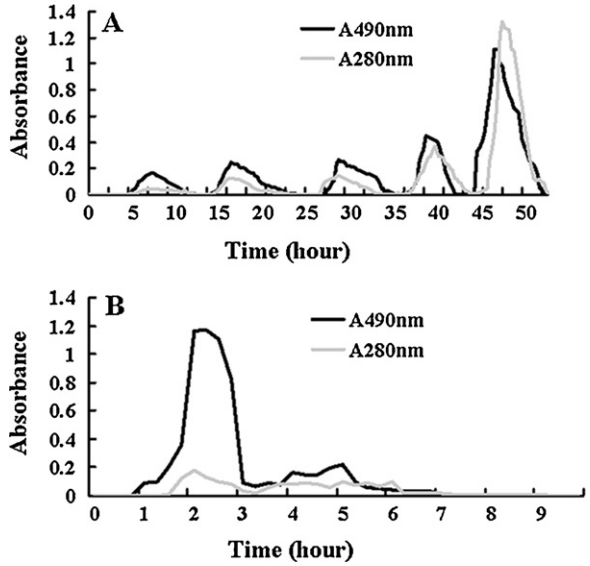

Fig. 2. Chromatography of LRGP1 on ion-exchange and size exclusion chromatography column. (A) Elution profile of the decolored polysaccharide LRP from *Lycium ruthenicum* on a DEAE-cellulose column (5 cm × 40 cm, HCO_3^- form). Eluent: distilled water, 0.05–0.50 mol/L $NaHCO_3$ solution; flow rate: 1.0 mL/min, 8 min/tube. LRP1, LRP2, LRP3, LRP4 and LRP5 were obtained by eluting with H_2O, 0.05, 0.10, 0.25, and 0.50 mol/L $NaHCO_3$, respectively. The carbohydrate content was determined by PhOH-H_2SO_4 acid method, and the relative protein content was monitored by UV absorption at 280 nm. The absorbance at 490 nm was that of the resulting solutions of polysaccharides after reacting with PhOH and H_2SO_4. (B) Elution profile of LRGP1 on a Sephadex G-100 column (1.5 cm × 100 cm). Eluent: 0.1 mol/L NaCl solution; flow rate: 0.5 mL/min, 5 min/tube.

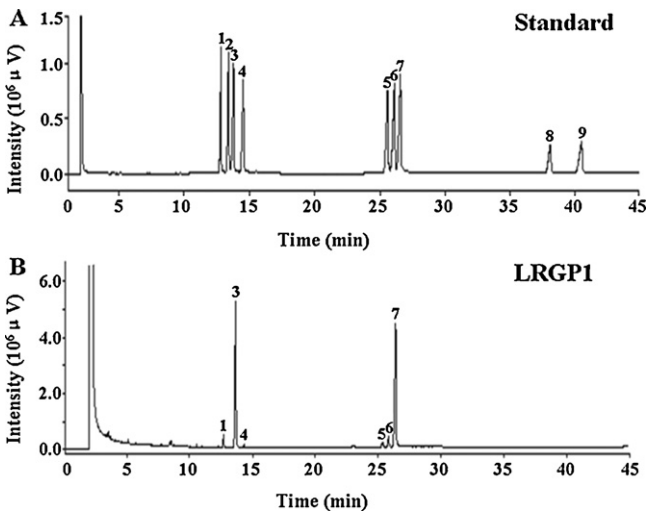

Fig. 3. Gas chromatography of standard monosaccharides and acid hydrolyzates of LRGP1. Column: rtx-5 ms (30 m × 0.25 mm × 0.25 μm); temperature: 180 °C (2 min)–6 °C/min, 240 °C (45 min); carrier gas: N_2 (0.6 mL/min). (A) Elution profile of alditol acetate derivatives of mixed standard monosaccharides. Peaks: (1) rhamnose, (2) fucose, (3) arabinose, (4) xylose, (5) mannose, (6) glucose, (7) galactose, (8) glucuronic acid, (9) galacturonic acid. (B) Elution profile of alditol acetate derivatives of acid hydrolyzates of LRGP1.

LRP1 was further purified by gel permeation chromatography on a Sephadex G-100 column. As shown in Fig. 2B, the main peak consisting of carbohydrate and protein was a glycoconjugate fraction. Fractions 6–13 were pooled, dialyzed and lyophilized, and LRGP1 was obtained with a yield of 0.003% of the crude herb.

3.2. Monosaccharide composition and character of LRGP1

LRGP1 was a water-soluble, white, and fluffy polysaccharide. Its aqueous solution has a neutral pH, and it was not soluble in organic solvents. Its monosaccharide composition is summarized in Table 1. The GC chromatogram of standard monosaccharides is shown in Fig. 3A, and the GC chromatogram of the monosaccharides from the *L. ruthenicum* sample is shown in Fig. 3B. By comparing retention times and areas of unknown peaks with those of the sugar standards, six monosaccharides including rhamnose, arabinose, xylose, mannose, glucose and galactose were identified in LRGP1 in the molar ratio of 0.65:10.71:0.33:0.67:1:10.41, and uronic acid was not detected. This result clearly demonstrated that both arabinose and galactose were the dominant monosaccharides in LRGP1.

As determined by PhOH–H_2SO_4 method, the total carbohydrate content was 96.2%, and protein was nearly absent (2.4%). A UV scan in the region of 200–400 nm showed strong absorbance at about 200 nm and weak absorbance at 280 nm, which further indicated that LRGP1 was a glycoconjugate. Compared with the untreated LRGP1, the alkali-treated LRGP1 sample had distinct absorbance at 240 nm, showing that β-elimination reaction had taken place. This demonstrated that the carbohydrate was linked to protein by O-linkage in LRGP1 (Lee, Cho, Song, Hong, & Yoo, 1996).

LRGP1 was scanned in the range of 4000–400 cm^{-1} for three or more times. In the FT-IR spectrum, signals in the regions of 3600–3200 cm^{-1}, 2929.1 cm^{-1} and 1647.5 cm^{-1} were due to O—H stretching vibration, C—H stretching vibration, and associated water, respectively. The peak at 1417.2 cm^{-1} displayed C—O stretching vibration; the absorption peaks between 1250 cm^{-1} and 950 cm^{-1} indicated that galactose conformation of LRGP1 was of the pyranose type (Ye, Qiu, Peng, Chen, Ye, & Lin, 2011); the weak absorbance at 895.2 cm^{-1} suggested that pyranoses existed in the β-configuration (Mathlouthi & Koenig, 1986). The IR spectrum showed no specific signature of uronic acid, corresponding with the data of GC analysis.

3.3. Homogeneity and molecular weight

The homogeneity and molecular weight of samples were determined by high performance gel permeation chromatography. The elution peak was single, symmetric and narrow, corresponding with the feature of homogeneous distribution. The calibration curve equation is: Log MW = 5.3685 − 1.7377*X*, R^2 = 0.9982, in which MW denotes the molecular weight of the standard dextran and *X* is the retention time. The molecular weight of LRGP1 was calculated to be 5.62×10^4 Da by this equation.

3.4. Partial acid hydrolysis

After Pronase E treatment, the glycan of LRGP1 was obtain, designated as LRGP1-OL, and its molecular weight was 5.49×10^4 Da by HPGPC. Based on the molecular weight of LRGP1 and LRGP1-OL, the protein moiety of LRGP1 might be expected to possess a molecular weight of about 1300 Da. LRGP1-OL was partially hydrolyzed with 0.02 mol/L H_2SO_4 and dialysis resulted in two subfractions of LRGP1-OL: non-dialyzable fraction LRGP1-OL-I (43.8 g% yield) and dialyzable fraction LRGP1-OL-O (56.2 g% yield). The sugar composition of these fractions is listed in Table 1. LRGP1-OL-I was composed of glucose and galactose in the molar ratio of 1:27. Based on HPGPC, its molecular weight was 2.42×10^4 Da. According to their molecular weight, monosaccharide composition and partial acid hydrolysis yield, the total molar ratio of LRGP1-OL was 2.5 times higher than that of LRGP1-OL-I. Compared with LRGP1-OL, the amount of arabinose of LRGP1-OL-I decreased considerably whereas the amount of galactose increased, suggesting that galactose residues present in the backbone which were not susceptible to mild acid hydrolysis, whereas arabinose residues were attached to the backbone as the branches and easy to be hydrolyzed. LRGP1-OL-I had no rhamnose, arabinose, xylose, or mannose, suggesting the presence of these monosaccharide residues in the branches of the polysaccharide.

For the dialyzable fraction LRGP1-OL-O, the sugar composition presented in Table 1 showed that arabinose residues were the predominant monosaccharide in the side chains, confirming the presence of galactose polymer backbone in the LRGP1-OL mainly substituted by the branches of arabinose residues.

3.5. Glycosidic linkage analysis

To obtain more information about the linkage between monosaccharides, LRGP1-OL was subjected to a GC–MS linkage analysis. The total ion chromatogram of LRGP1-OL and LRGP1-OL-I is shown in Fig. 4. The results of methylation analysis of LRGP1-OL and LRGP1-I are shown in Table 2.

Data from the methylation analysis indicated that LRGP1-OL had a highly branched structure. The amount of total terminal sugars was equal to that of branched sugars, indicating the completeness of methylation. The non-reducing termini were composed of arabinose, xylose, mannose, and glucose. Branches were present on galactosyl residues and on a trace of rhamnosyl residues. The xylosyl residues were composed of 2,3,4-Me_3-Xyl, which suggested that xylose was present in the pyranose form. The arabinosyl residues were composed of terminal-, (1 → 2)-linked and (1 → 5)-linked arabinose. The mannosyl residues and glucosyl residues were present at the non-reducing termini.

LRGP1-OL-I showed only five peaks by GC–MS and GC. Termini were composed of glucose and galactose. The branching unit of LRGP1-OL-I was (1 → 3,6)-linked Gal, which indicated that the backbone of LRGP1-I was composed of either (1 → 3)-linked Gal or

Table 1
Sugar composition of LRPG1, LRGP1-OL-I and LRGP1-OL-O.

Polysaccharide fraction	Sugar composition (%)					
	Rhamnose	Arabinose	Xylose	Mannose	Glucose	Galactose
LRGP1	2.7	45.1	1.4	2.8	4.2	43.8
LRGP1-I	–	–	–	–	3.6	96.4
LRGP1-O	4.8	76.2	2.4	4.7	4.9	7.0

–: not detected.

Table 2
The results of methylation analysis of LRGP1-OL and LRGP1-OL-I.

Peak number	Partially methylated sugar[a]	Deduced linkage[b]	Relative molar ratio	
			LRGP1-OL	LRGP1-OL-I
1	2,3,5-Me_3-Ara	Ara-(1-	10	–
2	2,3,4-Me_3-Xyl	Xyl-(1-	1	–
3	3,5-Me_2-Ara	-2)-Ara-(1-	12	–
4	2,3-Me_2-Ara	-5)-Ara-(1-	10	–
5	2,3,4,6-Me_4-Man	Man-(1-	2	–
6	2,3,4,6-Me_4-Glc	Glc-(1-	3	1
7	2,3,4,6-Me_4-Gal	Gal-(1-	–	6
8	3-Me-Rha	-2,4)-Rha	2	–
9	2,3,6-Me_3-Gal	-4)-Gal-(1-	4	–
10	2,4,6-Me_3-Gal	-3)-Gal-(1-	9	4
11	2,3,4-Me_3-Gal	-6)-Gal-(1-	4	10
12	2,4-Me_2-Gal	-3,6)-Gal-(1-	13	7

–: Not detected.
[a] Analyzed by GC–MS, after per-O-methylation, total acid hydrolysis, reduction, and acetylation. 2,3,5-Me_3-Ara = 1,4-di-O-acetyl-2,3,5-tri-O-methyl-arabinose, etc.
[b] Based on derived O-methylalditol acetates.

(1 → 6)-linked Gal. The amount of (1 → 3,6)-linked-Gal was equal to that of terminal galactose and glucose.

The structural features of type II arabinogalactans found in many AGP consist for the most part of (1 → 3)-linked galactose residues, some of which are substituted at O-6 with arabinose and galactose residues (Fincher, Stone, & Clarke, 1983; Goellner, Utermoehlen, Kramer, & Classen, 2011; Redgwell, Curti, Fischer, Nicolas, & Fay, 2002). The galactose residues of LRGP1-OL were mostly (1 → 3)-linked and (1 → 3,6)-linked, consistent with a galactan backbone of (1 → 3)-linked galactose substituted at O-6 with arabinose and galactose sidechains.

Compared with LRGP1-OL, LRGP1-OL-I had no terminal arabinose, terminal xylose, terminal mannose, (1 → 2)-linked Ara, (1 → 5)-linked Ara, (1 → 2,4)-linked Rha, and (1 → 4)-linked Gal, suggesting that they existed as branches. The number of (1 → 6)-linked Gal and terminal Gal of LRGP1-OL-I was increased, while the number of (1 → 3,6)-linked Gal and terminal Glc was decreased. As shown in Table 2, the increase of six (1 → 6)-linked Gal in LRGP1-OL-I was concomitant with the decrease of six (1 → 3,6)-linked Gal branching unit. The decrease of (1 → 3,6)-linked Gal was probably due to the hydrolysis at O-3 of galactose in 0.02 mol/L H_2SO_4 and the conversion of (1 → 3,6)-linked Gal to (1 → 6)-linked Gal. After partial acid hydrolysis, the increase in both terminal and (1 → 6)-linked galactose residues accompanied the loss of the terminal arabinose residues indicates that the terminal arabinose residues are attached to the O-3 position of the galactose in (1 → 6)-linked galactose sidechains. The decrease of (1 → 3)-linked Gal indicated that some (1 → 3)-linked Gal were located at sidechains. It can be deduced from the results that the backbone of LRGP1-OL was composed of (1 → 3)-linked Gal. Based on the above results, the

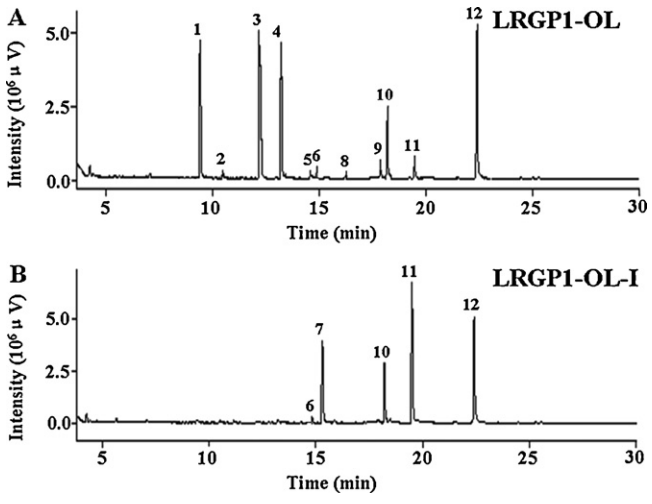

Fig. 4. GC/MS spectra of the methylation products LRGP1-OL and LRGP1-OL-I. (A) The total ion chromatogram of partially methylated alditol acetates of LRGP1-OL. (B) The total ion chromatogram of partially methylated alditol acetates of LRGP1-OL-I. Column: rtx-5 ms (30 m × 0.25 mm × 0.25 μm); temperature: 140 °C (2 min)–2 °C/min, 250 °C (30 min). Peaks: (1) 1,4-di-O-acetyl-2,3,5-tri-O-methyl-arabinose; (2) 1,5-di-O-acetyl-2,3,4-tri-O-methyl-xylose; (3) 1,2,4-tri-O-acetyl-3,5-di-O-methyl-arabinose; (4) 1,4,5-tri-O-acetyl-2,3-di-O-methyl-arabinose; (5) 1,5-di-O-acetyl-2,3,4,6-tetra-O-methyl-mannose; (6) 1,5-di-O-acetyl-2,3,4,6-tetra-O-methyl-glucose; (7) 1,5-di-O-acetyl-2,3,4,6-tetra-O-methyl-galactose; (8) 1,2,4,5-tetra-O-acetyl-rhamnose; (9) 1,4,5-tri-O-acetyl-2,3,6-tri-O-methyl-galactose; (10) 1,3,5-tri-O-acetyl-2,4,6-tri-O-methyl-galactose; (11) 1,5,6-tri-O-acetyl-2,3,4-tri-O-methyl-galactose; (12) 1,3,5,6-tetra-O-acetyl-2,3-di-O-methyl-galactose.

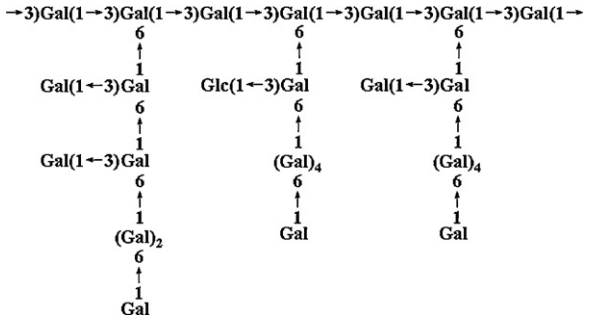

Fig. 5. The hypothetical structure of the repeat unit of LRGP1-OL-I.

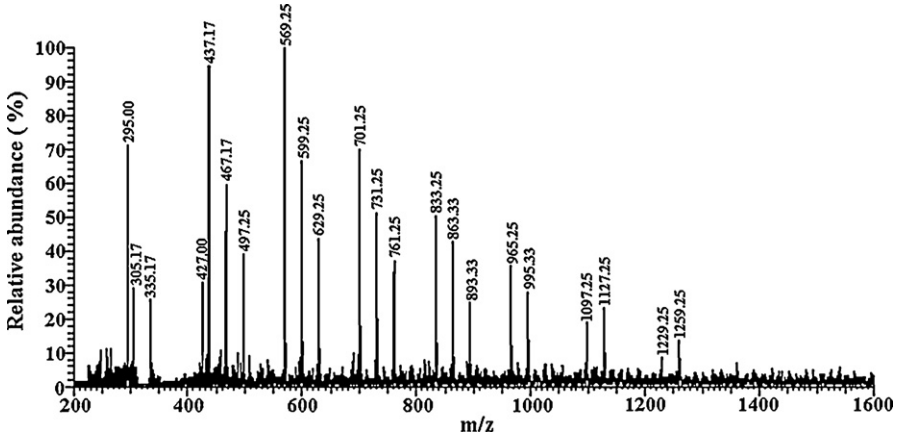

Fig. 6. Positive ion ESI-MS analysis of oligosaccharides from LRGP1-OL-O. Most molecular ion peaks were assigned to $[M + Na]^+$, whereas two peaks (m/z 295.00 and 427.00) were assigned to $[M + H]^+$.

structure of the repeating unit of LRGP1-OL was assigned to that shown in Fig. 5. However, this is only one of the possible structures, and the branches can be arranged in other reasonable orders.

3.6. ESI-MS analysis of the dialyzable oligosaccharides

The mass spectrum of LRGP1-OL-O revealed numerous peaks of oligosaccharide fragments (Fig. 6). Structures of the arabogalactan-derived oligomers were proposed, taking into consideration sugar composition, linkage data and molecular masses.

Ions at m/z 305.17, 437.17, 569.25, 701.25, 833.25, 965.25, 1097.25 and 1229.25, corresponding to $[M + Na]^+$ adducts of pentose polymers with degree of polymerization ranging from two to nine, respectively, suggest that LRGP1-OL side chains was in different degree of polymerization of arabinose, consisting with the results by GC. Only two oligosaccharides (m/z 295.00 and 427.00), corresponding to $[M + H]^+$ peaks of $DeoxyHexPent_1$ and $DeoxyHexPent_2$ separately, can be assigned as RhaAra and $RhaAra_2$. This further suggested the location of rhamnose at the terminal of side chains. In addition, ions at m/z 467.17, 497.25, 599.25, 629.25, 731.25, 761.25, 863.33, 893.33, 995.33, 1127.25, and 1259.25 can be ascribed to $[M + Na]^+$ adducts of $HexPent_2$, Hex_2Pent, $HexPent_3$, Hex_2Pent_2, $HexPent_4$, Hex_2Pent_3, $HexPent_5$, Hex_2Pent_4, $HexPent_6$, $HexPent_7$ and $HexPent_8$, respectively. LRGP1-OL-O contains large amount of hexose residues of mannose, galactose, and glucose, and the branches of LRGP1-OL contain abundant arabinose. Thus, these peaks probably originate from side chains composed of arabinose, rhamnose, mannan, galactose, and glucose.

4. Conclusion

Based on the monosaccharide composition analysis, partial acid hydrolysis, methylation analysis and ESI-MS analysis, we conclude that LRGP1 is a highly branched arabinogalactanprotein with a backbone of (1 → 3)-linked Gal. The backbone was partially substituted at O-6 of galactose residues by arabinose and galactose residues. The branches were composed of (1 → 5)-linked Ara, (1 → 2)-linked Ara, (1 → 6)-linked Gal, (1 → 3)-linked Gal, (1 → 4)-linked Gal and (1 → 2,4)-linked Rha. Arabinose, xylose, mannose, and glucose were located at the terminal of the branches. Thus, LRGP1 is different from LBGP in structure, and it may exhibit some special bioactivities. This is the first report on structural characterization of polysaccharide from *L. ruthenicum*. Further bioactivity investigation of LRGP is worth making in the future work for better exploiting this substance.

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