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Relationship between structure and immunological activity of an arabinogalactan from *Lycium ruthenicum*

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ABSTRACT

An immunologically active arabinogalactan (LRGP3) was selectively degraded by acetolysis, mild acid hydrolysis and enzymatic digestion. After *exo*-α-L-arabinofuranosidase digestion, 56% of the arabinosyl chains were released. The resistant product (LRGP3-AF) had markedly increased complement fixating activities. The acid hydrolysis product (LRGP3-T) contained (1 → 3)-linked (17.6%), (1 → 6)-linked (23.1%), (1 → 3,6)-linked (30.1%) and terminal (29.2%) galactosyl residues, and its complement fixating activity was lower than that of LRGP3-AF. The side chains (Oligo-S) consisted of arabinose, galactose, and rhamnose in the molar ratios 16.8:1.4:1.0. The complement fixating activity of Oligo-S was weak, but Oligo-S had potent macrophage stimulation activity. Degradation of arabinosyl residues in LRGP3 decreased the macrophage stimulation activity, but the galactan backbone still expressed partial activity. The results demonstrated that the galactan backbone of the polymer might be essential for the expression of complement fixating activity and the arabinosyl side chains could be more responsible for the macrophage activation activity. There may be several structurally different active sites involved in the immunological activity of LRGP3.

1. Introduction

Lycium ruthenicum Murr. is a nutritional food belonging to the family of Solanaceae, which is widely distributed in the salinized desert of northwestern China. *L. ruthenicum* is known for its functional properties, as it is high in anthocyanin, essential oils, organic acids, trace minerals, and polysaccharides. *L. ruthenicum* has been consumed as a fruit or been used as a raw material to prepare nutritious drinks. Active constituents of *L. ruthenicum* are reported to be have a variety of biological activities (Zheng et al., 2011). Of these ingredients, polysaccharide has been known as its major bioactive compound.

In a previous study, an arabinogalactan, LRGP3, was isolated from the fruits of *L. ruthenicum* (Peng et al., 2012). It was characterized as a highly branched polysaccharide with a backbone of (1 → 3)-linked β-D-galactopyranosyl residues, many of which were substituted at the O-6 position by galactosyl or arabinosyl groups. Many arabinogalactans have shown immunomodulatory activity, in some cases by activating the complement system and in other cases by enhancing the activity of macrophage (Choi, Kim, Kim, & Hwang, 2005; Classen, Thude, Blaschek, Wack, & Bodinet, 2006; Mellinger et al., 2008; Nergard et al., 2005). The structural features that have been related with immunomodulatory activity are the monosaccharide and glycosidic-linkage composition, conformation, molecular weight, functional groups, and branching characteristics (Ferreira, Passos, Madureira, Vilanova, & Coimbra, 2015). It has been reported the immunomodulatory activity of the arabinogalactan from Radix Astragali was associated to random coil conformation (Yin et al., 2012). Acetyl groups and sulfate groups of arabinogalactan have been considered to be important for expression of the macrophage stimulatory activity (Xie et al., 2008). Removal of arabinosyl side chains leads to a drop of the complement fixation activity showing that the branching regions may be involved in the bioactive site of the molecule (Diallo, Paulsen, Liljebäck, & Michaelsen, 2003).

The arabinogalactan LRGP3 has already been shown to stimulate macrophage *in vitro* (Peng, Liu, Shi, & Li, 2014). However, the bioactive domains of the polysaccharide are unknown. In the present study, we examined the structure–activity relationship by treating LRGP3 with acetolysis, partial acid hydrolysis and enzy-

Abbreviations: LRGP3, *Lycium ruthenicum* glycoconjugate polysaccharide 3; LRGP3-B, the backbone of LRGP3; LRGP3-AF, *exo*-α-L-arabinofuranosidase resistant fraction of LRGP3; LRGP3-T, mild trifluoroacetic acid hydrolysate of LRGP3; Oligo-S, oligosaccharide side chains of LRGP3; MWCO, molecular weight cut off.

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matic digestion, and the biological activities of the different structural domains of the polymer were compared.

2. Materials and methods

2.1. Materials

The fruit of *L. ruthenicum* was purchased from Jiahe Biological Engineering Cooperation (Qinghai, China). It was oven-dried at 50 °C and subsequently crushed into powder. The arabinogalactan (LRGP3), was isolated as described previously (Peng et al., 2012).

2.2. Chemicals

Bio-Gel P-30 and Bio-Gel P-4 were purchased from Bio-Rad Co. (USA); Dowex 50 WX8-400 cation exchange resin were from Sigma–Aldrich (St. Louis, MO, USA); Standard monosaccharides and T-dextran series of different standard molecular weights were purchased from Sigma Chemical Co. (USA); *exo*-α-L-arabinofuranosidase (*A. niger*) was purchased from Megazyme Co. (USA); LPS (*Escherichia coli* 055:B5) was purchased from Sigma–Aldrich (St. Louis, MO); RAW264.7 cell line was purchased from Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences; RPMI 1640 medium and fetal bovine serum were from Invitrogen-Gibco; other reagents used were of analytical reagent grade and supplied by Sinopharm Chemical Reagent Ltd. Co. (Shanghai, China).

2.3. General methods

The carbohydrate content was determined by the phenol–sulfuric acid method using glucose as a standard (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956). A Lambda 25 UV/VIS spectrophotometer (Perkin Elmer, USA) was used to detect the absorbance. The carbohydrate sample (2 mg) was totally hydrolyzed with 2 M trifluoroacetic acid (2 mL) at 120 °C for 2 h. The monosaccharide composition was determined by gas chromatography using the method described previously (Lehrfeld, 1985). GC was performed by a Shimadzu GC2010 equipped with a capillary column of rtx-5 m (30.0 m × 0.25 mm × 0.25 μm). The temperature program was: 180 °C for 2 min, raised 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. The molecular weight of glycans were determined by high performance gel permeation chromatography on a TSK-Gel G4000SW column, using a Waters Alliance 2414 refractive index detector (Alsop & Vlachogiannis, 1982). Macrophage viability was determined using the MTT assay (Mosmann, 1983).

2.4. Acetolysis

LRGP3 (60 mg) was acetylated by a mixture of acetic anhydride, acetic acid and sulfuric acid (10:10:1, v/v, 10 mL) at 20 °C for 18 h (Yalin, Cuirong, & Yuanjiang, 2006). The resulting solution was neutralized with sodium hydrogen carbonate. The acetolysate was de-O-acetylated by treatment with 0.2 M sodium methoxide in methanol, and the solution was deionised with Dowex 50 WX8-400 cation exchange resin. The resulting solution was applied to a Bio-gel P-30 column. The column was eluted with distilled water using a pump with a flow rate of 0.4 mL/min, and fractions of 4 mL were autocollected. The elution was monitored by the phenol–sulfuric acid method. Appropriate fractions were collected according to the carbohydrate profile of the eluants.

2.5. Digestion of LRGP3 with enzyme

LRGP3 was incubated with *exo*-α-L-arabinofuranosidase (0.8 U) in 50 mM acetate buffer (pH 4.0) at 40 °C for 10 h. The solution was boiled in a 100 °C water bath to stop the reactions. The digestion products was fractionated on Bio-gel P-30 column with distilled water, and the carbohydrate fractions eluted in the void volume (LRGP3-AF) and the included volume were collected. Each fraction was desalted by passing through Dowex 50 WX8-400 cation exchange resin and lyophilized for further analysis.

2.6. Partial acid hydrolysis

LRGP3 (70 mg) was treated with 0.1 M trifluoroacetic acid (10 mL) for 1 h at 80 °C. The resulting solution was neutralized with sodium hydroxide, and dialyzed against deionized water using a dialysis bag with a MWCO of 6000, giving rise to dialyzable (outside the dialysis bag) sample and non-dialyzable (inside the dialysis bag) sample. The non-dialyzable fraction was desalted using gel filtration chromatography on a Bio-gel P-30 column, giving the trifluoroacetic acid hydrolysis resistant fraction LRGP3-T. The dialyzable oligomers were designated as Oligo-S (oligosaccharide side chains). Oligo-S was analysed by ESI-MS, and further fractionated on Bio-gel P-4 column. Each fraction was desalted by passing through cation-exchange resin with Dowex 50 WX8-400 and evaporated to dryness.

2.7. Methylation analysis

Each sample was methylated by the Ciucanu and Kerek method (Ciucanu & Selvendran, 1984). The methylated samples were hydrolyzed with 2 M trifluoroacetic acid at 121 °C for 2 h, reduced with sodium borohydride, and then acetylated. The resulting alditol acetates were analyzed by GC and GC–MS. Peaks of methylated sugars were identified on the basis of relative retention times and fragmentation patterns. The calibration of molar ratios for each sugar were estimated from the peak areas and response factors (Sweet, Shapiro, & Albersheim, 1975). GC–MS was performed on a Shimadzu instrument GCMS-QP2010 gas chromatograph equipped with an electron impact ion source. The capillary column used was rtx-5 (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. 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.

2.8. ESI-MS analysis

The released oligosaccharides from LRGP3 were analyzed using a LTQ-XL ion-trap mass spectrometer equipped with an electrospray ion source and a HPLC system. Samples (1 mg) were loaded onto a Dowex 50 WX8-400 cation exchange column. The eluted fractions were collected and diluted 1000 times with MeOH for ESI-MS analysis. 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 (Thermo-Fisher, USA), 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. Data acquisition and processing were performed through the Xcalibur software.

2.9. Complement fixating assay

The complement fixation activity was measured by the inhibition of haemolysis of antibody sensitized SRBC by complement from human sera (Michaelsen, Gilje, Samuelsen, Høgåsen, & Paulsen, 2000). Samples were dissolved with veronal buffer. 50 μL of the sample solutions and 50 μL of the serum dilutions were added to wells in 96-well plate; then 50 μL of sensitized sheep erythrocytes were added and incubated for 30 min. After centrifugation, the supernatants (100 μL) were transferred to a microtiter plate and absorbance read at 405 nm. 100 μL of distilled water was mixed with 50 μL of sensitized sheep erythrocytes to obtain a value of 100% haemolysis. The mixture of veronal buffer, diluted serum and sensitized sheep erythrocytes was used as the medium control. Inhibition of lysis induced by the test sample was calculated by the formula:

$$[(A_{control} - A_{sample})/A_{control}] \times 100\%$$

2.10. Cell culture

Mouse macrophage cell line RAW264.7 was purchased from the Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences. Macrophages were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum, 100 units/mL penicillin and 100 units/mL streptomycin at 37 °C under 5% CO_2 and 95% air.

2.11. Macrophage activation assay

The *in vitro* macrophage stimulating activity was evaluated by measuring nitrite production of murine RAW264.7 macrophages. Nitrite was quantified by a spectrophotometric assay according to the Griess reaction (Green et al., 1982). RAW264.7 cells were seeded into 96-well plates at a density of 5×10^5 cells/mL in the media described above, and stimulated for 24 h in duplicates with samples (100 μg/mL), LPS (0.1 μg/mL) as positive controls, and medium alone. The level of NO was analyzed by a commercially available Kit (Beyotime Institute of Biotechnology, China) (Wang et al., 2010). Briefly, 50 μL of medium were mixed with 50 μL of Griess reagent in a 96-well plate. Nitrite concentration was determined by spectrophotometry (540 nm) from a standard curve derived from $NaNO_2$.

3. Results and discussion

3.1. Preparation of the backbone from LRGP3

LRGP3 consisted of arabinose, galactose, and rhamnose in a molar ratio of 14.9:10.4:1.0. In order to obtain the backbone of the arabinogalactan, LRGP3 was subjected to partial acetolysis. After acetolysis, a degraded polymer fraction (LRGP3-B) and oligosaccharide fraction were obtained by gel permeation chromatography on a Bio-gel P-30 column (Fig. 1). The elution profile of LRGP3-B was a single peak on a TSK-Gel G4000SW column (Fig. 2A), showing that it was a homogeneous polymer. The molecular weight of LRGP3-B was estimated to be 13.1 kDa, showing that the backbone of the polymer was not cleaved. LRGP3-B was composed of galactose as the only sugar, indicating that arabinosyl and rhamnosyl residues of side chains had been removed. Further detailed structural information of LRGP3-B was investigated by methylation analysis. Only one glycosyl residue of (1 → 3)-linked galactosyl residue was identified by GC–MS in LRGP3-B (Fig. 2B), showing that it was a linear (1 → 3)-linked galactan. LRGP3-B had no (1 → 3,6)-linked galactosyl residues, indicating that

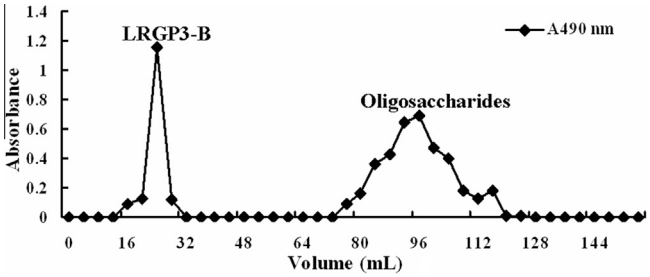

Fig. 1. Gel filtration chromatogram on of partial acetolysis products of LRGP3 on a Bio-gel P-30 column (1.5 cm × 80 cm). Eluent: distilled water; flow rate: 0.4 mL/min, 10 min/tube. The elution was monitored by the phenol–sulfuric acid method (490 nm).

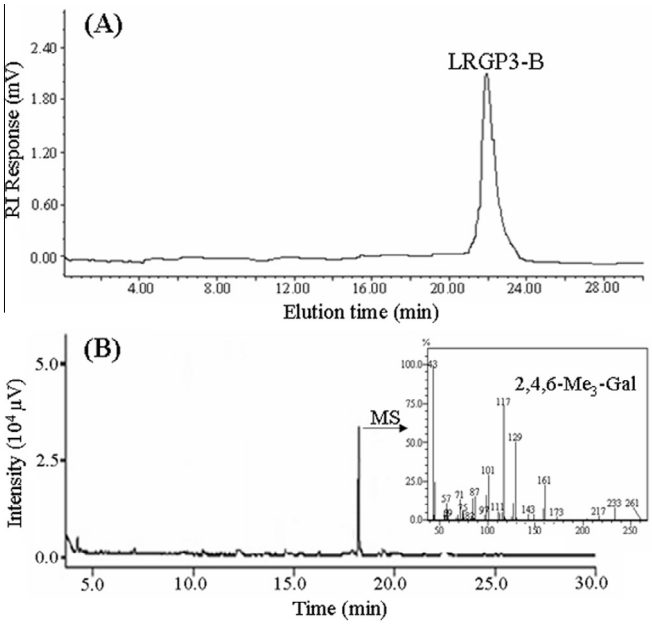

Fig. 2. Molecular weight and linkage analysis of LRGP3-B. (A) Elution profile of LRGP3-B on high performance liquid gel permeation chromatography. (B) Electron impact ionization mass spectra of the partially methylated alditol acetates of LRGP3-B. Peaks: 1,3,5-tri-O-acetyl-2,4,6-tri-O-methyl-galactose.

branched chains had been entirely cleaved by acetolysis. Partial acetolysis gave rise to disappearing of (1 → 3,6)-linked galactosyl residues and conversion of (1 → 3,6)-linked galactosyl residues to (1 → 3)-linked galactosyl residues. The result conformed to previous reports that (1 → 6)-linked glycosidic bond was easily cleaved by acetolysis (Rosenfeld & Ballou, 1974).

3.2. Enzymatic digestion of LRGP3

LRGP3 was digested with *exo*-α-L-arabinofuranosidase and the enzyme resistant fraction LRGP3-AF was isolated by gel filtration on Bio-gel P-30. Sugar composition of the enzyme nonresistant fraction indicated that the only monosaccharide released was arabinose. LRGP3-AF was composed of arabinose, galactose, and rhamnose in the molar ratios 0.6:1.0:0.1. Digestion of LRGP3-AF with *exo*-α-L-arabinofuranosidase did not hydrolyse all of the arabinosyl residues, and the decreased content of arabinose corresponded to 56% of the arabinose in LRGP3. Methylation analysis of LRGP3-AF showed that the non-reducing terminal and (1 → 5)-linked arabinosyl residues were decreased remarkably (Table 1), whereas a large proportion of (1 → 2)-linked arabinosyl residues still remained after the enzymic digestion. The (1 → 2,4)-linked

Table 1
The results of methylation analysis of the products obtained after degradation of LRGP3.

Methylated sugar[a]	Deduced linkage[b]	Molar ratio			
		LRGP3	LRGP3-B	LRGP3-AF	LRGP3-T
2,3,5-Me_3-Ara	Ara(1→	15.1	–	5.7	–
3,5-Me_2-Ara	→2)Ara(1→	18.9	–	27.8	–
2,3-Me_2-Ara	→5)Ara(1→	22.6	–	–	–
2,3,4,6-Me_4-Gal	Gal(1→	1.9	–	11.3	29.2
2,4,6-Me_3-Gal	→3)Gal(1→	18.5	100	24.8	17.6
2,3,4-Me_3-Gal	→6)Gal(1→	5.7	–	8.4	23.1
2,4-Me_2-Gal	→3,6)Gal(1→	13.2	–	16.2	30.1
3-Me-Rha	→2,4)Rha(1→	4.1	–	–	
2,3Me_2-Rha	→4)Rha(1→	–	–	5.8	

–: Not detected; LRGP3: *Lycium ruthenicum* glycoconjugate polysaccharide 3; LRGP3-B: the backbone of LRGP3; LRGP3-AF: *exo*-α-L-arabinofuranosidase resistant fraction of LRGP3; LRGP3-T: mild trifluoroacetic acid hydrolysate of LRGP3; Oligo-S: oligosaccharide side chains of LRGP3.

[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.

rhamnosyl residues were disappeared concomitant with the appear of (1 → 4)-linked rhamnosyl residues, indicating that some arabinosyl residues were attached to the O-2 position of rhamnosyl residues. Compared with LRGP3, LRGP3-AF contained more terminal galactosyl residues. These results suggested that the arabinosyl side chains attached to galactosyl residues in LRGP3 were partially removed by the digestion with arabinofuranosidase.

3.3. Preparation of oligosaccharide side chains released from LRGP3

In order to study the contributions of oligosaccharide side chains to the biological activity of LRGP3. LRGP3 was partially hydrolyzed using mild conditions (0.1 M trifluoroacetic acid, 80 °C, 1 h) and dialyzed, which gave rise to the acid resistant fraction (LRGP3-T) and the oligosaccharide side chains fraction (Oligo-S). LRGP3-T was all composed of galactose, suggesting that all of the arabinosyl residues and rhamnosyl residues were released. The result showed that arabinosyl residues were as the branches and easy to be hydrolyzed. This was in accordance with previous observations which showed arabinosyl residues are easily hydrolysed than galactosyl residues (Redgwell, Curti, Fischer, Nicolas, & Fay, 2002). In addition, all of the rhamnosyl residues were released by mild acid hydrolysis, further suggesting rhamnosyl residues located near the terminal of side chains.

Methylation analysis suggested that LRGP3-T contained less (1 → 3)-linked, (1 → 3,6)-linked galactosyl residues and more (1 → 6)-linked than LRGP3 (Table 1). This was in accordance with previous reports that (1 → 6)-linked hexopyranosyl residues were more stable than other linkage types to acid hydrolysis (Redgwell et al., 2002). The increase in both terminal and (1 → 6)-linked galactosyl residues following mild acid treatment indicated that arabinosyl side chains were attached to the position 3 of the (1 → 3,6)-linked galactosyl residues. Oligo-S consisted of arabinose, galactose, and rhamnose in the molar ratios 16.8:1.4:1.0. Electrospray ionization mass spectrometric analysis was performed on oligosaccharide side chains liberated from LRGP3 (Fig. 3). Combining MS results with monosaccharide composition, the *m*/*z* ions 305.08, 437.17, 569.25, 701.25, 833.33, 965.33 corresponded to $[M+Na]^+$ for arabinan oligomers with their degree of polymerization values ranging from 2 to 7, respectively. The ESI-MS data showed the low stability of arabinosyl oligomers towards acid hydrolysis. The peaks found at *m*/*z* 203.08 and 319.17 were assigned to monosaccharide (Gal) and disaccharide (Rha-Ara) respectively. The hydrolysis cleaved small amounts of galactosyl

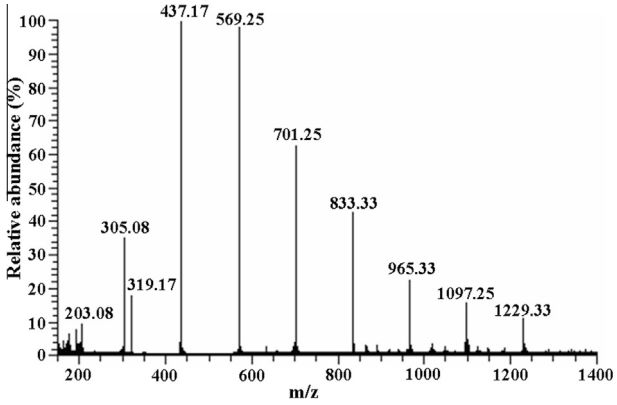

Fig. 3. Positive ion ESI-MS spectra of oligosaccharides from Oligo-S. The molecular ion peaks were assigned to $[M+Na]^+$.

residues which were released as monosaccharides. The scheme for sequential degradation of LRGP3 was done as set out in Fig. 4. These different structural region samples were employed for further activity study.

3.4. Complement fixating activity of the carbohydrate units

The complement fixating activities of LRGP3 and its hydrolysates are shown in Fig. 5A. All the high molecular weight samples, LRGP3, LRGP3-AF, LRGP3-T and LRGP3-B, retained potent dose-dependent complement fixating activities. LRGP3 was digested with *exo*-α-L-arabinofuranosidase, and the enzyme resistant fraction (LRGP3-AF) had markedly increased complement fixating activities. The increased activity was due to the removal of arabinose. Therefore, the arabinose contributed less to the complement fixating activity of LRGP3. Removal of the arabinosyl side chains from LRGP3 increased the complement fixating activities, suggesting that the inner galactan core of LRGP3-AF makes a major contribution to the complement fixating activity of LRGP3. After mild acid treatment, the complement fixating activity of the product (LRGP3-T) was lower than that of arabinofuranosidase digested product LRGP3-AF. It was postulated that the (1 → 3)-linked galactosyl residues, which were attached to the (1 → 6)-linked galactan in side chains, may be involved in the expression of the complement-activating activity of LRGP3. The activity of the backbone fraction (LRGP3-B) was potent, indicating that the (1 → 6)-linked galactan backbone might be essential for the expression of activity. The oligosaccharide side chains fraction (Oligo-S) had weak activities, showing that the arabinosyl side chains in LRGP3 were less involved in the expression of the complement activating activity.

3.5. Contributions of structural domains to the macrophage stimulation activity

LRGP3 has shown potent macrophage stimulation activity (Peng et al., 2014), therefore the structure and activity relationships was investigated. Nitric oxide is a good marker for macrophage activation evaluation, and it is transformed into nitrite in the medium, which can be measured by the Griess method. The macrophage stimulation activity of LRGP3 and its degradation products was examined (Fig. 5B). Possible LPS contamination of the samples were excluded using Tachypleus amebocyte lysate assay (obtained from Chinese Horseshoe Crab Reagent Manufactory, CO., Ltd, Xiamen, China), with the LPS concentration lower than 0.1 EU/μg. RAW264.7 cells were treated with a series of concentrations (0, 12.5, 25, 50, 100 and 200 μg/mL) of samples for 24 h. These

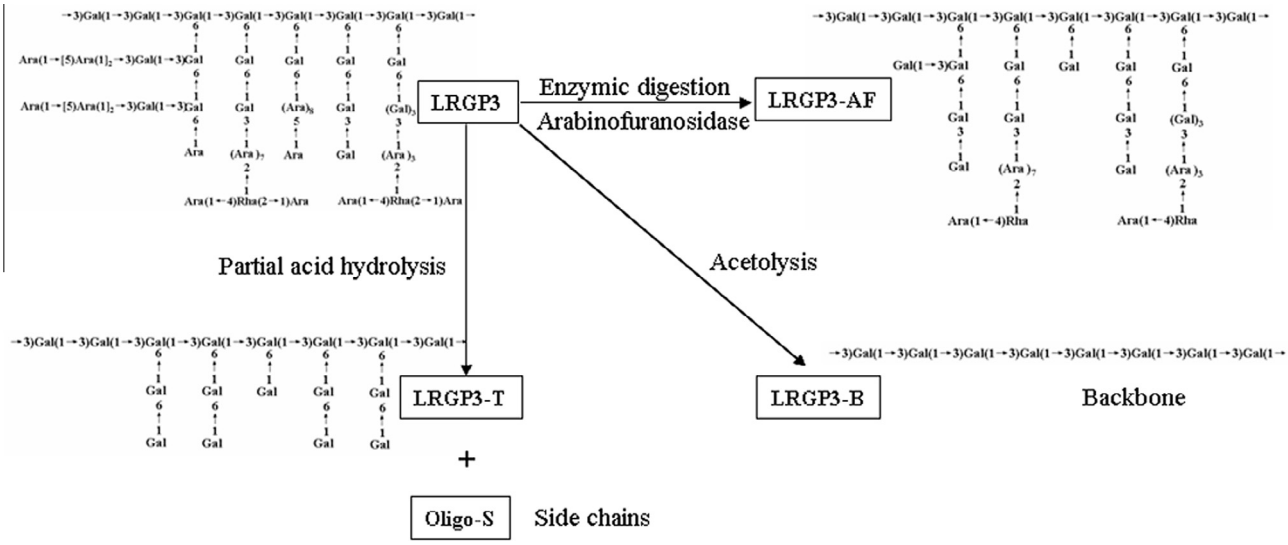

Fig. 4. Scheme of the procedure for sequential degradation of LRGP3.

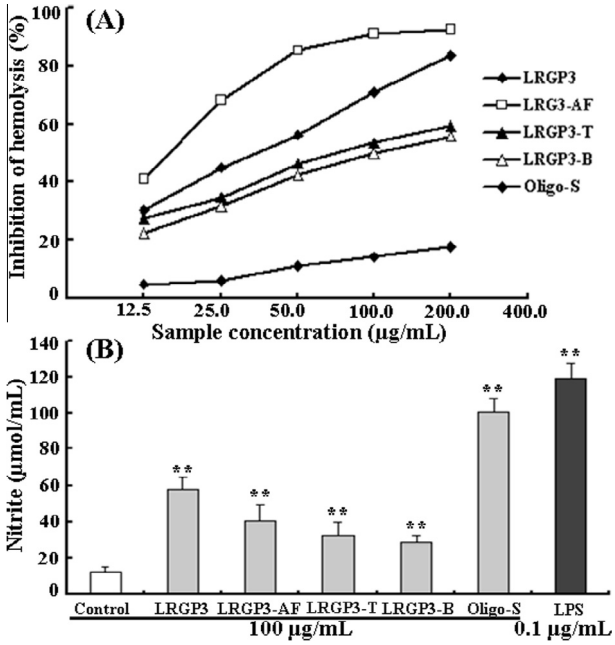

Fig. 5. Immunological activity of LRGP3 and its degradation products. (A) Complement fixation activity of LRGP3 and its degradation products. (B) Macrophage stimulating activities of the original arabinogalactan (LRGP3) and the products obtained after treatment of LRGP3 with *exo*-α-L-arabinofuranosidase (LRGP3-AF), mild acid hydrolysis (LRGP3-T, Oligo-S), acetolysis (LRGP3-B).

samples did not affect RAW264.7 cell viability at concentrations of less than 200 μg/mL. Of the carbohydrate units from LRGP3, Oligo-S was more active than all other samples. The activity of arabinofuranosidase resistant fraction (LRGP3-AF) was slightly less than that of the original arabinogalactan (LRGP3). These result indicated that the arabinosyl side chains of the polymer could be more responsible for the macrophage activation activity. The nitrite production stimulation by LRGP3-B was similar to that of LRGP3-T, indicating that the (1 → 3,6)-linked galactosyl residues in the backbone were not important for the macrophage activation of LRGP3. Therefore, the macrophage stimulation activity of LRGP3 may be due to a combination of the arabinosyl side chains and the galactan backbone.

4. Conclusions

In this study, various treatments were applied to LRGP3 to investigate its structure and immunological activity relationship. Among the structural domains of LRGP3, the galactan backbone of the polymer might be essential for the expression of complement fixating activity and the arabinosyl side chains could be more responsible for the macrophage activation activity. LRGP3 is a highly complex arabinogalactan and the immunocompetent domains seem to be located both in the galactan backbone and arabinosyl side chains. There may be several structurally different active sites involved in the immunological activity of LRGP3. These results provide certain theoretical guidance for further research on the structure–activity relationship and potential practical applications of *L. ruthenicum* polysaccharides in the food industry.

Acknowledgements

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