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Structural characterization of a bioactive water-soluble heteropolysaccharide from *Nostoc sphaeroids* kütz

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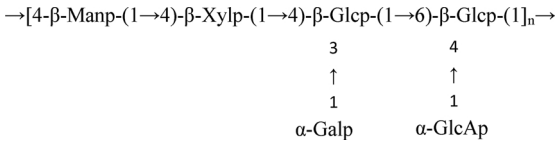
ABSTRACT

A water soluble *Nostoc sphaeroids* kütz polysaccharide (NSKP) was extracted and purified by precipitating hot-water extract from *Nostoc sphaeroids* kütz with 40% (v/v) ethanol. The yield was 40.10% (w/w), and weight average molecular weight (M_w) was determined as 1.31×10^5 g/mol. Cell culture study showed that NSKP could stimulate the NO release from RAW264.7 macrophages. The ρ value (R_g/R_h) of NSKP was calculated as 1.50, indicating flexible random coils conformation of NSKP in aqueous solution. According to methylation analysis, and 1D & 2D NMR spectra, the molecular structure of polysaccharide NSKP was deduced as below:



1. Introduction

Nostoc sphaeroids kütz, commonly known as Ge-Xian-Mi or Tian-Xian-Mi in China, is a lower unicellular blue-green algae, belonging to the Nostocaceae of the family Cyanophyta (Whitton & Potts, 2000; Xia & Gao, 2002). It has long been used as medicinal consumption, such as nyctalopia, archoptoma, scald, and antipyretic (Deng, Yan, Hu, & Hu, 2008; Hao et al., 2011). According to previous studies, *Nostoc sphaeroids* kütz is high in polysaccharides, protein, soluble vitamins and some other nutrients (Chen, Wang, & Pan, 2003; Mo, Zhou, Wang, & Xie, 2007).

Recent studies demonstrated that polysaccharides extracted from *Nostoc sphaeroids* kütz played important roles in enhancing immune, anti-tumor, anticoagulant, and antibacterial activities (Chang, Li, Deng, & Zhao, 2009; Peng, 2012; Zhu et al., 2014). It was reported that the activities of polysaccharides could be affected by their structures (Lee, Takeshita, Hayashi, & Hayashi, 2011; Liu et al., 2016; Yin, Nie, Zhou, Wan, & Xie, 2010). Therefore, it is important to analyze the structure of polysaccharides for further understanding the structure-activity relationship. The previous studies on *Nostoc sphaeroids* kütz polysaccharides were focused on extraction, purification, and biological activity (Mo, Xie, Wang, & Liu, 2004; Mo, 2011); however, the corresponding molecular structure and conformation were seldom reported and still remained unclear. Several published articles on structure analysis of polysaccharide fractions isolated from *Nostoc* species including *Nostoc commune*, *Nostoc carneum*, *Nostoc flagelliforme* revealed that most of the purified polysaccharides from these materials were complex heteroglycans. They were mainly comprised of glucose,

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mannose, arabinose, galactose, xylose, rhamnose, ribose, galacturonic acid and glucuronic acid with different combinations and types of glycosidic linkages (Brüll et al., 2010; Helm et al., 2000; Jensen et al., 2010, 2013; Kanekiyo et al., 2005). Some of them had common structural features with similar residues; however, the detailed structures were different due to the differences on the species, various environmental factors, and purification methods. In order to provide defined structure information for future studies, a water-soluble polysaccharide fraction was purified from *Nostoc sphaeroids* kütz, and the structure and bioactivity were characterized using methylation analysis, 1D & 2D NMR spectroscopy, high-performance size exclusion chromatography, and cell culture, respectively. The work will provide more information for understanding the structural characteristics of *Nostoc sphaeroids kütz* polysaccharide.

2. Materials and methods

2.1. Materials

Nostoc sphaeroids kütz was provided by Hunan Yandi Bioengineering Co. Ltd. N-(3-Dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (EDC), lipopolysaccharide (LPS), and monosaccharide standards (i.e. rhamnose, arabinose, galactose, glucose, xylose, mannose, fucose, glucuronic acid) were purchased from Sigma-Aldrich (USA). DMEM, RPMI-1640, fetal bovine serum (FBS), and pancreatin were purchased from Gibco company (USA). Trifluoroacetic acid (TFA) was purchased from Merck company (USA). RAW 264.7 cell line was purchased from the Institute of Chinese Academy of Sciences (Beijing, China). All other reagents were of analytical grade.

2.2. Isolation and purification of Nostoc sphaeroids kütz polysaccharide

The dried and crushed *Nostoc sphaeroids* kütz powder was extracted with 500 volumes of distilled water for 2 h at 100 ℃ to get water soluble polysaccharide. The extract was centrifuged at 9000 g for 15 min. The supernatant was collected and concentrated to one-tenth of its original volume followed by precipitation in 40% (v/v) ethanol at 4 ℃ for overnight. The precipitate was collected by centrifuging at 9000 g for 15 min and washed with 45% (v/v) ethanol alternately for four times. After that, the precipitate was dissolved in 10 volumes of distilled water and then precipitated in 40% (v/v) ethanol at 4 ℃ overnight. After centrifugation at 9000 g for 15 min, the precipitate was collected and rinsed with 45% (v/v) ethanol twice, and then the precipitate was freeze-dried to obtain fraction designated as NSKP. The content of total sugar was determined using the phenol-sulfuric acid method (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956).

2.3. Determination of homogeneity and molecular weight of NSKP

The homogeneity and weight average molecular weight (M_w) of NSKP were estimated by high-performance size exclusion chromatography (HPSEC) using the Waters HPLC 2695 system equipped with a UV and a refractive index (RI) detector for concentration determination, a multiple angle laser light scattering detector (MALLS) for direct molecular weight determination, and a differential pressure viscometer (DP) for viscosity determination.

The analysis columns were composed of a guard column (TSK PWXL, Japan) and a TSK G6000 PWXL (7.8 mm × 30 cm, Japan) column in series eluting with 0.15 M $NaNO_3$ and 0.05 M NaH_2PO_4 containing 0.02% (w/w) NaN_3 (pH = 7) at the flow rate of 0.5 mL/min. The column and RI detector temperature were maintained at 30.0 ℃. The wavelength of MALLS was 623.8 nm, and the refractive index increment (*dn/dc*) was set to be 0.146 mL/g.

2.4. Monosaccharide composition analysis

NSKP sample (2 mg) was hydrolyzed with 3 mL trifluoroacetic acid (2 M, TFA) at 110 ℃ for 4 h. Determination of monosaccharide composition of polysaccharide was performed by a high performance anion exchange chromatography (HPAEC) system (Dionex ICS-2500) equipped with a CarboPac™ PA20 column (3 mm × 150 mm) and a pulsed amperometric detector (Dionex, USA). The data was analyzed by Chromeleon chromatography management system software (Dionex, USA). NaOH (2 mM) and MilliQ water were used as the mobile phase with a flow rate of 0.45 mL/min. The composition and content of monosaccharides of NSKP were detected by comparing retention time and peak area of the tested residues with monosaccharide standards.

2.5. FT-IR analysis

The transmission FT-IR spectrum of NSKP was recorded by IR spectrometer (Thermo Fisher Scientific, USA) using the KBr disk method and scanned from 4000 to 400 cm^{-1}.

2.6. Carboxyl reduction of the acidic polysaccharide NSKP

The reduction of uronic acid residues in NSKP was conducted according to the method described by previous paper (Liu, Zhang, & Han et al., 2014; Liu, Zhang, & Tang et al., 2014; Liu et al., 2016). Briefly, NSKP (40 mg) was dissolved in 20 mL distilled water, and then 500 mg EDC was added. The pH value was maintained at 4.75 for 2.5 h with 0.04 M HCl. Then, 16 mL of 2 M fresh $NaBH_4$ solution was slowly added into the reaction flask for 1 h with pH controlled at 7 for 2 h with 4 M HCl. Finally, the reaction mixture was dialyzed against distilled water (MWCO 3500 Da), and the retentate was freeze-dried as NSKP-R.

2.7. Methylation and GC–MS analysis

Methylation of NSKP and NSKP-R were conducted to analyze the linkage patterns of polysaccharide based on previous study (Guo, Cui, Wang, & Young, 2008). Firstly, the dried samples (2 mg) were dissolved in 0.5 mL DMSO by magnetic stirring at 85 ℃ for 2 h, and then 20 mg NaOH was added into the bottle and stirred at 30 ℃ for 3 h. Methyl iodide (0.3 mL) was added and stirred at 30 ℃ for 2.5 h in dark place. Subsequently, deionized water was added to stop reaction. The solution was extracted by methylene chloride and water. The organic phase was washed with deionized water for 4 times, and then dried by nitrogen to obtain the methylated polysaccharide. Complete methylation was confirmed by the disappearance of the −OH band (3100-3700 cm^{-1}) in the IR spectrum. Secondly, the permethylated products were hydrolyzed with 0.5 mL 4.0 M trifluoroacetic acid (TFA) for 6 h at 100 ℃. The hydrolysate was reduced with 3 mg sodium borodeuteride ($NaBD_4$) for 12 h at 30 ℃, followed by acetylating at 100 ℃ for 2 h with 0.5 mL acetic anhydride. Thirdly, the partially methylated alditol acetates (PMAA) were analyzed by gas chromatography-mass spectrometry (Thermo Finnigan TRACE 2000/MS) system, equipped with a DB-5MS column (30 m × 0.25 mm × 0.25 μm). The temperature program increased from 180 to 270 ℃ at 20 ℃/min, with holding at 270 ℃ for 25 min. The individual peaks of the PMAAs and fragmentation patterns were identified by their mass spectra and relative retention time in GC. The percentage of methylated sugars was calculated by the peak areas.

2.8. NMR analysis

NSKP sample (30 mg) was dissolved into 1 mL D_2O and then lyophilized, repeated three times. The 1D NMR (^{1}H-NMR and ^{13}C-NMR) and 2D NMR (COSY, TOCSY, HSQC, HMBC and NOESY) spectra were recorded at 70 ℃ using a Bruker VNMRS 600 NMR spectrometer. ^{1}H chemical shifts were referenced to residual HDO at δ 4.29 ppm (70 ℃) as internal standard and ^{13}C chemical shifts were acquired in relation to