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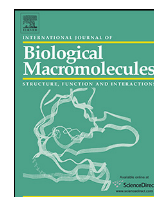

Structure analysis of a heteropolysaccharide from fruits of *Lycium barbarum* L. and anti-angiogenic activity of its sulfated derivative

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ABSTRACT

Angiogenesis plays a crucial role in tumor growth and development. Blocking angiogenesis of tumor cells has become one of the most promising approaches in cancer therapy. Here, an acidic polysaccharide designated LBP1B-S-2 with an average molecular weight of 80.00 kDa, was extracted and purified from dried mature fruits of *Lycium barbarum* L. by DEAE Sepharose^{TM} Fast Flow and Sephacryl S-300 HR columns. Monosaccharide composition analysis indicated that the LBP1B-S-2 was composed of rhamnose, arabinose, galactose and glucuronic acid in a molar ratio of 3.13: 53.55: 39.37: 3.95. The backbone of LBP1B-S-2 was consisted of 1, 3-linked β-D-Gal*p*, 1, 6-linked β-D-Gal*p* and branches contained 1, 4-linked β-D-Glc*p*A, T-linked β-D-Gal*p*, 1, 6-linked β-D-Gal*p*, T-linked α-L-Ara*f*, T-linked β-L-Ara*f*, 1, 5-linked α-L-Ara*f* and T-linked β-L-*Rha*p directly or indirectly attached to C-3 position of 1, 6-linked β-D-Gal*p* or C-6 position of 1, 3-linked β-D-Gal*p*, according to the results of partial acid hydrolysis analysis, methylation analysis, IR and NMR spectra. The sulfated derivative of LBP1B-S-2, could significantly inhibit the tube formation of human microvascular endothelial cells (HMEC-1) *in vitro* at concentration of (95 nM) without significant cytotoxicity.

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1. Introduction

Angiogenesis is a physiological process of new capillaries from pre-existing vasculature [1,2]. It is now widely recognized as an essential step of tumor growth, invasion and metastatic [3,4]. Due to its essential role in cancer, controlling tumor-associated angiogenesis is a modern and promising strategy in limiting tumor progression [5].

Lycium barbarum L. is a kind of multi-branched shrub belonging to the genus *Lycium* of the family solanaceae, and it is widely distributed in arid and semi-arid regions of Northwestern China, Southeastern Europe and the Mediterranean areas [6]. Orange-red fruit of *Lycium barbarum* is also called as wolfberry or Gouqizi. As a well-known traditional Chinese herbal medicine and valuable nourishing tonic, the dried fruit of *Lycium barbarum* L. has been used historically in China and other Asian countries as antipyretic, and for nourishing liver and kidney, moistening lung, anti-inflammation and improving eyesight for more than thousands of years [7]. As we know, the compositions of traditional Chinese medicines are complex. Previous researches indicated that polyphenols, polysaccharides, flavonoids, carotenoids, saponins and polypeptides build a compound library of *Lycium barbarum* fruit [8,9]. At present, polysaccharide as one of the major bioactive ingredient has been proved to possess a wide array of bioactivities including anti-tumor properties [10], anti-aging [11], stimulating immunogenicity [12], antioxidant properties [13], treatment or prevention of cardiovascular and metabolic disorders [14], neuroprotection [15], etc [16].

In the past decades, many studies have been proved that polysaccharides play an active role in the growth and development of living organisms due to a wide range of biological activities and relatively low toxicity [17]. The structural improvement and molecular modification of polysaccharides have been become a very important strategy to achieve strong and diverse bioactivities [18]. Sulfated modification of polysaccharide is an effective way to augment or change its biological activities [19]. Thus, discovery and identification of sulfated derivatives of polysaccharides fractionated from natural herbs with strong biological activities and relatively low cytotoxicity were valuable for researchers and potential beneficial for patients.

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In this paper, we report structure of an acidic polysaccharide LBP1B-S-2 from *Lycium barbarum* and sulfation modified polysaccharide (Sul-LBP1B-S-2), and its anti-angiogenesis effect.

2. Experimental

2.1. Materials and reagents

The dried mature fruits of *Lycium barbarum* (5.0 kg) were purchased from Shanghai Kangqiao Chinese Medicine Tablet Co., Ltd. DEAE Sepharose™ Fast Flow and Sephacryl S-300 HR columns were obtained from GE Healthcare. Food-grade cellulase (U = 2000), food-grade papain (U = 400000) and food-grade amylase (U = 1000000) were purchased from Shandong Lonct Enzymes Co., Ltd. and Nanning Pangbo Biological Engineering Co., Ltd., respectively. CMC (1-Cyclohexyl-3-(2-morpholinoethyl) carbodiimide metho-*p*-toluenesulfonate) was purchased from TCI, Tokyo, Japan. PMP (1-phenyl-3-methyl-5-pyrazolone) and TFA (trifluoroacetic acid) were from sigma-Aldrich, USA. Monosaccharide standards were from Fluka, Switzerland. Dextran standards were obtained from Pharmacia Co., Sweden. All other reagents not mentioned were purchased as analytical grade.

2.2. Extraction, isolation and purification of LBP1B-S-2

The dried mature fruits of *Lycium barbarum* (5.0 kg) were firstly crushed into powder. Then the powder (1.0 kg) was immersed in 20 L water and then treated with cellulase (30 g), papain (5 g) and amylase (10 g) under stirring at 55 °C for 1 h, followed by enzyme inactivation by heating the solution at 100 °C. After filtered and centrifugation, the extract solution was dialyzed against running water using dialysis membrane (MWCO 3500 Da) for three days, followed by addition of five volumes of 95% EtOH to concentrate retentate for overnight. The precipitate was washed with absolute ethanol and acetone alternately for three times, and then freeze-dried to obtained the crude polysaccharide designated LBP1 (129 g/5 kg, yield is 2.58%).

The brownish crude polysaccharide LBP1 (6 g) was dissolved in 60 mL distilled water and centrifuged. The supernatant was fractionated by anion-exchange chromatography on a DEAE Sepharose™ Fast Flow column and eluted stepwise with distilled water, 0.05, 0.1 and 0.2 M NaCl solution. The fraction of LBP1 B was pooled from the 0.1 M NaCl eluent. Subsequently, LBP1 B was further purified by gel permeation chromatography Sephacryl S-300 column (100 cm × 2.6 cm), which was eluted with 0.2 M aqueous NaCl to give the target polysaccharide LBP1B-S-2 (112 mg, yield is 1.87%).

2.3. Homogeneity and molecular weight

Homogeneity of polysaccharide was examined by HPGPC (high-performance gel permeation chromatography) using the Agilent 1260 HPLC system fitted with the GPC software, equipped with the UV and RI detectors and two tandem columns (UItrahydrogel ™ 2000 and 500). For molecular weight estimation, the columns were calibrated by a series of dextran standards with different molecular (T-700, T-580, T-500, T-110, T-80, T-70, T-40, T-11, T-9.3, T-4 series). Moreover, the column temperature was kept at 40.0 °C, 0.1 mol/L $NaNO_3$ was used as an eluant, and the flow rate maintained at 0.5 mL/min. All the samples were prepared as 2 mg/mL solutions with eluant and further centrifuged, and 20 μL of the supernatant was injected to be analyzed.

2.4. Monosaccharide composition analysis

The glycosyl composition was analyzed using PMP pre-column derivation method as precious reported [20]. In briefly, LBP1B-S-2 (2 mg) was hydrolyzed with 4 mL 2 M TFA (trifluoroacetic acid), followed by PMP derivation. 20 μL of the derivative solution was injected into HPLC to be analyzed.

2.5. FT-IR spectrum

The IR spectra were determined based on the previous report [21]. 2 mg polysaccharide (native polysaccharide or sulfated polysaccharide) was ground with dried KBr powder and pressed into pellets, then scanned from 4000 to 600 cm^{-1} for the analysis.

2.6. Linkage pattern analysis

Methylation of the polysaccharide (LBP1B-S-2) was carried out based on previous methods to analysis the linkage patterns of polysaccharides [22,23]. Briefly, the sample LBP1B-S-2 (10 mg) was dried overnight with phosphorus pentoxide (P_2O_5), then 2 mL DMSO was added into reaction bottle under nitrogen. Subsequently, dried sodium hydroxide powder (60 mg) was added and stirred for 3 h. Methyl iodide (0.5 mL) was added dropwise into the reaction bottle for 30 mins under ice bath and then another 0.5 mL methyl iodide was added into reaction bottle for further methylation for another 2 h at room temperature in dark place. Finally deionized water (2 mL) was added to quench the reaction. The reaction solution was extracted by 15 mL chloroform and 15 mL water (1:1, v/v), and the organic phase was washed with deionized water for three times, and then air dried to obtain the methylated polysaccharide. After repeating the procedure for four times, the fully methylated products were converted into partially methylated alditol acetate (PMAA). PMAAs and analyzed through gas chromatography–mass spectrometry (GC–MS).

2.7. Partial acid hydrolysis

LBP1B-S-2 (200 mg) was hydrolyzed with 0.2 M TFA (20 mL), and incubated at 100 °C for 1 h. The solution was evaporated with methanol to remove TFA completely under reduced pressure, and then dialyzed against distilled water (MWCO 3500 Da) for 24 h. Finally, the retentate LBP1B02I (62 mg) were obtained. LBP1B02I was further hydrolyzed with 1 M TFA (10 mg/mL) at 100 °C for 1 h to yield the LBP1B1I.

2.8. NMR analysis

For NMR analysis, LBP1B-S-2 (50 mg) was exchanged and dissolved into 0.45 mL D_2O (99.8% D), and then lyophilized and re-dissolved into 0.45 mL D_2O (99.8% D). The 1D and 2D NMR spectra (COSY, HSQC and HMBC) were measured at 25 °C with acetone as internal standard (δ_H = 2.29, δ_C = 31.5). NMR spectra were recorded on a Bruker AVANCE III NMR spectrometer.

2.9. Preparation of sulfated polysaccharide

LBP1B-S-2 (100 mg) was dissolved in 5 mL dried formamide and stirred at room temperature. Meanwhile, sulfation reagent made from pyridine (2 mL) and chlorosulfonic acid (6 mL) (1:3, v/v) was added dropwise. The reaction mixture was continually stirred at 60 °C for 3 h. Then, the mixture was neutralized with 5 M NaOH followed by ice-cooled. Then the neutralized solution was dialyzed against saturated $NaHCO_3$ (sodium bicarbonate) for 24 h firstly and then against distilled water for 3 days. The retentate was

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