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Structure and immunobiological activity of a new polysaccharide from *Bletilla striata*

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ABSTRACT

A new polysaccharide (designated BSPF2), with a molecular weight of 2.35×10^5 Da, was isolated from the tubers of *Bletilla striata*. It contained mannose, glucose, and galactose in a molar ratio of 9.4:2.6:1.0. The acetyl content of BSPF2 was estimated to be 2.9%, and acetyl groups were located in positions 3 and 6 of mannosyl residues. The structural features were elucidated by a combination of monosaccharide composition analysis, periodate oxidation, partial acid hydrolysis, acetolysis, and methylation analysis. The results indicated that the backbone of BSPF2 consisted of (1 → 4)-linked mannosyl residues and (1 → 4)-linked glucosyl residues in a molar ratio of 2:1. About three fifths of glucosyl residues in the backbone were branched at O-6 position, and the terminal sugar residues were composed of mannosyl residues. Immunological assay results demonstrated that BSPF2 significantly induced the spleen cell proliferation in a dose-dependent manner.

1. Introduction

Bletilla striata (Thunb.) Reichb.f. belongs to the Orchid family. It is mainly distributed in China, North Korea, Japan and Burma. The tubers of *B. striata* is a well-known traditional Chinese herb and is used to treat swollen tissues, skin cracks, freckles, burns and abscesses. Pharmacological researches have shown that herbal polysaccharides are an important functional factor in traditional Chinese medicine. The tubers of *B. striata* contains a lot of polysaccharide. It was reported that *B. striata* polysaccharide could induce the proliferation of human umbilical vascular endothelial cells and vascular endothelial growth factor (Wang et al., 2006). Furthermore, it was also found that *B. striata* polysaccharide could act on macrophage and simulate cells to expression some proinflammatory cytokines (Diao et al., 2008). However, there are little reports on the structural features of *B. striata* polysaccharide. An early publication reported that a *B. striata* polysaccharide contained mannose and glucose in a molar ratio of 3:1, with the backbone consisting of (1 → 4)-linked aldohexopyranosyl residues (Tomoda, Nakatsuka, Tamai & Nagata, 1973). Another *B. striata* polysaccharide was reported to be composed of mannose and glucose in a molar ratio of 2.4:1.0, with the molecular weight of 1.35×10^5 Da (Wang et al., 2006). These results indicated that *B. striata* polysaccharide contain more than one kind of fractions.

In the present study, a novel polysaccharide fraction (BSPF2) was isolated from the tubers of *B. striata*. The structural features of BSPF2 was elucidated by monosaccharide composition, periodate oxidation, partial acid hydrolysis, acetolysis, and methylation analysis. Further, the immunobiological activity of BSPF2 was analyzed by ^{3}H-thymidine incorporation method.

2. Materials and methods

2.1. Plant material

The tubers of *Bletilla striata* was purchased from Huike Botanical Development Cooperation, PR China. The plant material was oven-dried at 60 °C and subsequently crushed into powder.

2.2. Chemicals

Standard monosaccharides and T-dextran series of different standard molecular weights were purchased from Sigma Chemical Co. (USA); DEAE-Cellulose-52 was purchased from Yuanye biological technology Co. (Shanghai, China); Bio-gel-P-300 was purchased

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from Bio-Rad Co. (USA); Dowex 50 WX8-400 cation exchange resin was purchased from Sigma–Aldrich (St. Louis, MO, USA); LPS (*Escherichia coli* 055:B5) was purchased from Sigma–Aldrich (St. Louis, MO); other reagents used were of analytical grade and supplied by Sinopharm Chemical Reagent Ltd. Co., (Shanghai, China).

2.3. Analytical methods

The carbohydrate content was determined by the phenol-sulfuric acid method using dextran 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). A UV scan in the region of 200–400 nm was performed on the spectrophotometer. Starch contamination was estimated by treatment with pancreatic α-amylase (Asamizu & Nishi, 1979). The homogeneity and molecular weight of samples 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). Acetyl groups were located according to the method of De-Belder (De Belder & Norrman, 1969).

2.4. Isolation and purification of B. striata polysaccharide

The *B. striata* tubers powders (1000 g) was extracted with acetone at 50 °C for 4 h. After filtration, the residue was suspended in 5000 mL distilled water and extracted for 2 h at 80 °C. After suction filtration, the extraction was similarly repeated. All extraction solutions were pooled and concentrated with a rotary evaporator at 40 °C. A 4-fold volume of ethanol was added to the extracts. The precipitate was treated with Sevag reagent (Navarini et al., 1999) to remove proteins and dialyzed against distilled water for 48 h. After lyophilization, crude *B. striata* polysaccharide (CBSP) was obtained. CBSP was dissolved in distilled water at 45 °C with continuous stirring and the solution was adjusted to pH 9.0 with aqueous ammonia. After stirring for another 4 h, the solution was neutralized with 1 mol/L HCl followed by dialysis and lyophilization. This decolorized CBSP (1.0 g) was dissolved in distilled water and applied to a anion-exchange column (6.0 cm × 55.0 cm) of DEAE-Cellulose-52. The column was first washed with distilled water, and then eluted with a step gradient (0.05, 0.10 and 0.15 mol/L NaCl) at a flow rate of 1 mL/min. Sugar-positive fractions were combined, concentrated, dialyzed against distilled water, and lyophilized. The obtained fractions were designated as BSP1, BSP2, BSP3 and BSP4, respectively. BSP2 (80 mg) was further purified by gel permeation chromatography on a Bio-Gel P-300 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. Then the purified polysaccharide BSPF2 was obtained.

2.5. Determination of monosaccharide composition

Polysaccharides (2 mg) was hydrolyzed with 2 mol/L trifluoroacetic acid (2 mL) 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-5ms (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.

2.6. Periodic acid oxidation and Smith degradation

BSPF2 was oxidized with 0.04 M $NaIO_4$ in the dark at 4 °C until stabilization of periodate consumed (Ghosh et al., 2008; Hu, Kong, Yang & Pan, 2011). The excess periodate was destroyed by adding ethylene glycol, and the solution was dialyzed against distilled water for 2 d. After it was reduced with $NaBH_4$ at 25 °C for 12 h, the residue was subjected to complete hydrolysis with 2 mol/L trifluoroacetic acid. The acid was removed by co-distillation with methanol under vacuum. Finally, the products were acetylated and analyzed in GC by using the same method as described in Section 2.5.

2.7. Selective hydrolysis of the branched polysaccharide

BSPF2 (80 mg for each hydrolysis) was dissolved in 10 mL of 0.02 mol/L H_2SO_4 and heated at 80 °C for 8 h and 12 h (Peng et al., 2012). The resulting solution was neutralized with NaOH and dialyzed against distilled water using membranes with size exclusion 3.5 kDa, and retained polymeric samples were lyophilized, giving rise to HP8 and HP12 fractions, respectively.

BSPF2 (80 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 (Wu, Sun & Pan, 2006). The resulting solution was neutralized with sodium hydrogen carbonate and was further purified on a Sephadex G-10 column, giving the partially acetylated polysaccharide AP1 and oligosaccharide fractions AP2. All the above samples were submitted to sugar composition analysis and methylation analysis.

2.8. Methylation analysis

Samples were methylated using modified Ciucanu method as described previously (Needs & Selvendran, 1993). The methylation procedure was repeated three times. Next, the samples were hydrolyzed with 2 mol/L trifluoroacetic acid (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. The capillary column used was rtx-5ms (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.

2.9. Spleen cells proliferation assay

Balb/C mice were sacrificed. The spleen was aseptically removed and minced through a 40 μm nylon cell strainer to achieve single cell suspension. Red blood cells were depleted with Tris-NH_4Cl lysis buffer. 5×10^5 spleen cells were stimulated with PSPF2 at serial concentrations, including 10, 50, 100 μg/mL. LPS (5 μg/mL) was positive control for the proliferation of the spleen cells. The cells were cultured in RPMI-1640 medium, supplemented with 10% FBS, penicillin (100 U/mL) and streptomycin (100 μg/mL) at 37 °C in a 5% CO_2 humidified incubator for 72 h and were pulsed with ^{3}H-thymidine (1 μCi/well) for the last 18 h. The cells were harvested on glass fiber filters using a Filtermate cell harvester (Packard). The amount of ^{3}H-thymidine incorporated into cells was measured using a β-scintillation counter (BECKMAN LS6500). The results are