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Structural characterization of an acidic Epimedium polysaccharide and its immune-enhancement activity

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ABSTRACT

One acidic polysaccharide named EPS-1 was isolated from the aqueous extract of the leaves of *Epimedium acuminatum* Franch. It may be composed of 1,4-linked α-D-Gal*p*A, 1,3,4-linked α-D-Gal*p*A, 1,6-linked β-D-Gal*p* and terminal α-L-Rha*p* residues in a molar ratio of 11.0:1.0:1.0:1.0 by chemical and spectroscopic analysis. EPS-1 possessed immune modulation effects on peripheral T lymphocyte and immature chBM-DCs such as promoting the proliferation and cytokine secretion of these cells and increasing the phagocytosis ability of immature chBM-DCs.

1. Introduction

Polysaccharides, one kind of natural biological macromolecules widely distributed in animals, plants, and microorganisms, possess many biological activities such as antioxidant (Cheng et al., 2013a, 2013b), antiviral (Chen et al., 2015), antimicrobial (Cheng et al., 2013a, 2013b), and immunomodulation (Meng et al., 2014). These bioactive polysaccharides have attracted oceans of attentions and become ideal candidates for extensively research.

Genus *Epimedium* (Berberidaceae) contains fifty-two species, some of which have been used as an important traditional Chinese medicine called "yin-yang-huo" to nourish "the kidneys", replenish 'yang' and strengthen the bones in East Asia for two thousand years (Cheng et al., 2013a, 2013b). The aerial parts of *Epimedium acuminatum* Franch., one of the medicinal species of *Epimedium*, have been recorded with potential effects of immunity enhancement (Sun, Hu, Wang, Zhang, & Liu, 2006), antioxidant (Cheng et al., 2013a, 2013b) and antimicrobial (Cheng et al., 2013a, 2013b). Our previous studies revealed that Epimedium polysaccharides (EPS) and their chemical derivatives showed significant effects in stimulating macrophages and T-lymphocytes, and played important roles as an immune adjuvant (Sun et al., 2006; Lu, Wang, Hu, Huang, & Wang, 2008).

In this paper, one polysaccharide named EPS-1 was isolated from the aqueous extract of the leaves of *E. acuminatum* Franch. Its

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preliminary structural characterization was detected by chemical composition analysis, UV, Fourier transform infrared spectroscopy (FT-IR), nuclear magnetic resonance (NMR) and scanning electron microscope (SEM). Moreover, its immunologic activities on lymphocyte and immature dendritic cells (DCs) of chicken were evaluated, which may provide a theoretical basis for further investigations on the structure-activity relationships of EPS.

2. Materials and methods

2.1. Materials and reagent

2.1.1. Plant material

The leaves of *E. acuminatum* Franch. were purchased in November, 2013 from Tianyuan Pharmacy in Jiangsu Province of China, where a voucher specimen (No. EP20130901) was deposited in Institute of Traditional Chinese Veterinary Medicine, Nanjing Agricultural University.

2.1.2. Reagents and instruments

Roswell Park Memorial Institute 1640 (RPMI-1640, Gibco, USA) was supplemented with 2% benzylpenicillin (100 IU/mL), 2% streptomycin (100 IU/mL) and 10% fetal bovine serum (FBS, Hyclone). Dulbecco's modified eagle medium (DMEM, Sigma, USA) was supplemented with 2% benzylpenicillin (100 IU/mL), 2% streptomycin (100 IU/mL) and 10% FBS. Phytohemagglutinin (PHA, Sigma, USA) was dissolved into 0.1 mg/mL with RPMI-1640. Lipopolysaccharide (LPS, Sigma, USA) was dissolved into 0.05 mg/mL with RPMI-1640. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Amresco Co.) was dissolved into 5 mg/mL with calcium and magnesium-free (CMF) PBS (pH 7.4). All of the reagents were filtered through 0.22 μm syringe filters.

Cellulose DEAE-52 and Sephacryl S-300 HR were purchased from Aoqing Chemical Reagent Co. (Nanjing, China). D-galactose, L-rhamnose, L-arabinose, D-glucose, D-xylose, D-mannose, D-fructose, D-glucoturonic acid and D-galacturonic acid were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Dextran series were purchased from National Institute of Metrology (NIM, Beijing, China). PHA and LPS solution were stored at −20 °C, MTT solution was kept at 4 °C in dark bottles. Dimethyl sulfoxide (DMSO) was purchased from Shanghai Lingfeng Chemical Co. (Shanghai, China). Lymphocyte separation medium was manufactured by Shanghai Huajing Biology Inc. (Shanghai, China). The other chemicals and reagents were analytical grade from Nanjing Shoude Chemical Co. (Nanjing, China).

UV spectra were detected on a Shimadzu UV-2401 spectrometer (Shimadzu, Japan). IR spectrum was performed with KBr pellets on a Shimadzu 8400S FT-IR infrared spectrometer (Shimadzu, Japan). HPGPC was measured on an Agilent 1100 series system equipped with a TSK-GEL G4000SW column (7.8 mm × 300 mm) and an Agilent RID-10A refractive index detector (Agilent, America). GC-MS was measured by Agilent GC 7890N/MSD 5973N (Agilent, America) with a DB-1701 (14% cyanide propyl-phenyl) methyl polysiloxane capillary column (30 m × 0.25 mm, film thickness 0.25 mm). HPLC was performed on Agilent 1100 system with a DIKMA Inertsil ODS-3 column (4.6 mm × 150 mm, 5 μm, Dikma, Japan) and detected by UV–vis DAD detector. NMR spectra were carried out on a Bruker AMX-500 (Bruker, Switzerland) with tetra-methylsilane (TMS) as an internal standard. SEM was examined with a SEM system (JSM-7500, JEOL, Japan).

2.2. Methods

2.2.1. Extraction, isolation and purification of EPS-1

The dried leaves of *E. acuminatum* Franch. (3 kg) were crushed and extracted with 95% EtOH-water (5 L, v/v) under reflux twice (2 h each time), then decocted with 24 L water three times (2 h each time). The filtered extract was combined and concentrated, then precipitated by dehydrated alcohol (4 volumes) to give the crude polysaccharide. The crude polysaccharide was dissolved in distilled water and deproteinized five times by Sevag method (chloroform: butanol 4:1, v/v) (Sevag, Lackman, & Smolens, 1938). The deproteinized polysaccharide was precipitated, decolorized and dialyzed with deionized water for 2 days, then lyophilized to give the total EPS (EPS_t).

EPS_t (1 g) was dissolved in 400 mL distilled water to extract polysaccharides EPS_{30}, EPS_{50} and EPS_{80} by stepwise precipitation method. Briefly, the polysaccharides were precipitated by adding ethanol up to the ethanol concentration of 30% (EPS_{30}), 50% (EPS_{50}) and 80% (EPS_{80}) at 4 °C for 12 h, then collected by centrifugation, washed twice with 95% ethanol, lyophilized and stored, respectively. EPS_{50} was dissolved in distilled water and subjected to a DEAE-Cellulose-52 column (5 cm × 30 cm), eluted with H_2O, 0.5 M NaCl and 1 M NaCl successively at a flow rate of 1 mL/min. The eluents (5 mL each) were monitored by the phenol-sulphuric acid method at 490 nm to give three different eluents curves. The same tubes were combined to yield EPS_{50}-A, EPS_{50}-B and EPS_{50}-C. EPS_{50}-B was concentrated and desalted on Sephadex G-50 (3 cm × 20 cm) using distilled water, collected and monitored by the above method. The same tubes were combined and lyophilized to give EPS_{50}-B1. EPS_{50}-B1 was further separated by a Sephacryl-S200HR column (3 cm × 30 cm) with 0.01 M $NaHCO_3$ following the methods before. The same tubes were combined and lyophilized to obtain EPS-1.

2.2.2. Purity, homogeneity and molecular weight of EPS-1

Proteins and nucleic acids were detected using a Shimadzu UV-2401 spectrometer (Shimadzu, Japan) between 500 and 190 nm (Hu, Liang, & Wu, 2015). The homogeneity and molecular weight was determined by HPGPC instrument with an Agilent 1100 HPLC system equipped with a TSK-GEL G4000SW column (7.8 mm × 300 mm) and an Agilent RID-10A refractive index detector. The column was calibrated by dextran of known molecular weights (5000, 12,000, 25,000, 50,000, 80,000 and 150,000 Da from NIM, Beijing, China) at 30 °C by distilled water, eluting at 0.5 mL/min with 1.6 MPa.

2.2.3. Analysis of chemical composition

Total sugar content was detected by phenol-sulfuric acid colorimetric method using glucose as standard (Hu et al., 2015). Protein content was determined by Bradford's method (Bradford, 1976). Uronic acid content was measured by vitriol-carbazole method using D-galacturonic acid as standard (Cheng et al., 2013a, 2013b). Total phenols content was analyzed by Folin–Ciocalteau assay (Sabir & Rocha, 2008). Configurations of sugars were determined using the Gerwig method (Hu et al., 2015).

2.2.4. Monosaccharide composition analysis

EPS-1 was hydrolyzed with trifluoroacetic acid (TFA, 2 M) in a round flask in a nitrogen atmosphere at 100 °C for 2 h (Li, Zhang, Wang, & Jiao, 2015). After repeated co-distillation with ethanol, the residues were treated with 1-phenyl-3-methyl-5-pyrazolone (PMP) by given method (Hu et al., 2015). The aqueous residue was filtered through 0.45 μm nylon membrane then analyzed by Agilent 1100 HPLC system with a DIKMA Inertsil octadecylsilyl-3 (ODS-3) column (4.6 mm × 150 mm, 5 μm, Dikma, Japan) with mixed acetonitrile and 0.1 M phosphate buffer (20:80, v/v, pH 6.9) at 1.0 mL/min at room temperature, detected by UV–vis DAD detector with a Chemstation system at 245 nm. Standard sugars were detected by the same methods. The sugar compositions were identified by comparing retention times with standard sugars, and the