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Characteristics and immune-enhancing activity of pectic polysaccharides from sweet cherry (*Prunus avium*)

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ABSTRACT

Two water soluble polysaccharides components PAPS-1 and PAPS-2 with homogeneously distributed molecular weight were obtained from *Prunus avium*. PAPS-1 and PAPS-2 contained GalA: Ara: Gal: Rha: GluA: Glu in 49.38: 32.39: 10.68: 4.66: 1.94: 0.48 and 77.18: 14.91: 3.39: 3.46: 0.93: 0.19 M ratios respectively, as well as trace amount of mannose and fucose. Infrared spectroscopy (IR), nuclear magnetic resonance (NMR) and methylation analysis indicated that both fractions were type I rhamnogalacturonan (RG-I) pectic polysaccharides with glycan side chains constituted mainly of arabinose with minor amount of galactose. Galacturonic acid methylation and sugar acetylation was found in both PAPS-1 and PAPS-2. Both PAPS-1 and PAPS-2 significantly induced the NO release from RAW264.7 cells and the expression of several immune-related molecular (TNFα, IL6, IL10, GCSF, iNOS, COX-2) was induced in RAW264.7 cells.

1. Introduction

Polysaccharides had been found to possess a wide range of bioactivities, such as immune regulating activity (Sun, Gao, Xiong, Huang, & Xu, 2014), antioxidant activity (Song, & Tang, 2016), antitumor activity (Chihara, Maeda, Hamuro, Sasaki, & Fukuoka, 1969). Plants are a rich source of polysaccharides which function mainly as either structural or energy storage components. Several studies have indicated that polysaccharides from plants have immune-enhancing activity (Ferreira, Passos, Madureira, Vilanova, & Coimbra, 2015; Zhang, Qi, Guo, Zhou, & Zhang, 2016).

Sweet cherry (*Prunus avium*) is one of the most popular fruits containing various bioactive components, such as phenolics (Cao et al., 2015), dietary fiber (Nawirska & Kwasniewska, 2005), melatonin (González-Gómez et al., 2009). Among these, the phenolics have been the most studied compounds in sweet cherry and found to be beneficial for their antitumor (Olsson, Gustavsson, Andersson, Nilsson, & Duan, 2004) and antioxidant properties in humans (Kelebek & Selli, 2011: Liu et al., 2001). There have, however been few health-related studies on sweet cherry polysaccharides, which have been reported to be mainly composed of pectins, hemicelluloses and cellulose (Nawirska & Kwasniewska, 2005). Only a few studies on their bioactivity have been carried out and structural studies have been limited to the monosaccharides composition (Basanta, Pla, Stortz, & Rojas, 2013; Salato, Ponce, Raffo, Vicente, & Stortz, 2013). According to the pervious research, sweet cherry might have a beneficial effect on the immune system in animals (Kelley, Rasooly, Jacob, Kader, & Mackey, 2006). As polysaccharides are known to be good immunomodulators, and sweet cherry is a popular fruit, the effects on the immune system of their polysaccharides is worthy of further study.

Macrophage is one of the important components of non-specific immunity, which play an important role in regulating innate and adaptive immune responses via the production of cytokines, such as tumor necrosis factor α (TNFα), interleukin 6 (IL6), interleukin 10 (IL10), and interferon γ (IFNγ). Macrophages also regulate immune responses by releasing inflammatory molecules such as nitric oxide (NO) (Commins, Borish, & Steinke, 2010; Le Page, Génin, Baines, & Hiscott, 2000; Medzhitov, & Janeway, 2000). RAW264.7 cells are macrophage-like cells that can easily be activated by immune-enhancing reagents. The activated RAW264.7 cells are able to release NO, followed by the release of a series of immune-related cytokines, which can be easily detected. Thus, RAW264.7 cells are commonly used as model for the in vitro evaluation of immune regulatory activity (Simas-Tosin et al., 2012; Zhu, et al., 2012).

In the present study, the water-soluble polysaccharides were isolated and classificated from *Prunus avium* and their structural characteristics and the immune regulatory active in vitro using RAW264.7 cells were studied.

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2. Materials and methods

2.1. Materials and reagents

The fruit of *Prunus avium* cv. Black pearl was collected at commercial maturity from the breeding nursery of Yantai Agricultural Sci & Tech Institute, Shandong Province. Disease and mechanical damage-free fruits with uniform shape and color were pitted and frozen quickly in liquid nitrogen, then stored at −80 °C for later use.

Cell culture media were the products of Gibco BRL (Gaithersburg, MD, USA). Lipopolysaccharide (LPS) and polymyxin B was purchased from Sigma-Aldrich Co. LLC. (St. Louis, MO, USA). NO detection kit and Bradford protein quantization kit were purchased from Beyotime Institute of Biotechnology (Jiangsu, China). Elisa kits for mice TNFα, IL6 and IL10 quantization were purchased from R&D Systems China Co. Ltd. (Shanghai, China). The primers for qRT-PCR were synthesized by Invitrogen Co. Ltd. (Carlsbad, CA, USA). HCl-methanol solution was purchased from the Xiya Reagent Co. Ltd. (Sichuan, China). Chromatographically pure 1-Phenyl-3-methyl-5-pyrazolone (PMP), glucose, galactose, arabinose, rhamnose, fucose, xylose, galacturonic acid, and glucuronic acid were purchased from Jingchun Bio-Chem Technology Co. Ltd. (Shanghai, China). Acetonitrile and trifluoroacetic acid (TFA) were of chromatographic grade from Sigma-Aldrich Co. LLC. (St. Louis, MO, USA). All other chemicals used were of analytical grade and purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China).

2.2. Isolation and purification of polysaccharides

The pulp of cherry fruit (600 g) was mixed with 3.5 L absolute ethyl alcohol and homogenized. The homogenate was placed at −20 °C for 24 h and then filtered. The residue was extracted twice (2h each time) with 80% ethanol in a solid-liquid rate of 1:20 (m:v) and filtered. The residue was subsequently refluxed with 300 mL ethanol to further remove the ethanol-soluble impurities. The residue was dried and extracted three times with hot water at 90 °C (2h each time). The filtrates were combined and concentrated ten times in a rotary concentrator. Then, ethyl alcohol was added slowly to the concentrated filtrate to a final concentration of 80%, and maintained at 4 °C for 24 h to fully precipitate the crude polysaccharide.

The precipitate was collected by centrifugation, and washed subsequently with ethanol, acetone and absolute ether. After removing all the organic solvents by drying, the residue was dissolved in ddH_2O (20 mg/mL) and subjected to ion-exchange chromatography using DEAE-Sepharose Fast Flow (1.6 × 40 cm, GE Co., Pittsburgh, PA, USA), eluting subsequently with ddH_2O, 0.05, 0.1, 0.2, 0.3 and 0.4 mol/L NaCl (3 column bed volume for each gradient) at a flow rate of 1 mL/min. The eluent was collected in 8 mL fractions per tube. All the fractions were measured by phenol-sulphuric acid method (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956) and results were expressed as a histogram. The peaks with high polysaccharide content was collected, concentrated at 45 °C and dialyzed for 48 h, following by lyophilization to obtain the polysaccharides.

The polysaccharides fractions were dissolved in 0.2 mol/L NaCl solution (10 mg/mL) for further purification using a HiPrep 26/60 Sephacryl S200 HR column (GE Co., Pittsburgh, PA, USA). NaCl solution (0.2 mol/L) was used as elution solvent, with flow rate of 1 mL/min. The eluent was collected in 4 mL aliquots per tube. All the fractions were measured by the phenol-sulphuric acid method (Dubois et al., 1956). The peaks with high polysaccharide content were collected, concentrated at 45 °C and dialyzed for 48 h, following by lyophilization to obtain the purified polysaccharides.

2.3. General analytical methods

2.3.1. Purity assessment

The polysaccharide content was determined by the phenol-sulphuric acid method (Dubois et al., 1956) and the total phenolics impurities in the polysaccharides were determined by the Folin-Ciocalteu method (Zhang et al., 2008). The total protein content in the polysaccharide was determined following the instruction for the Bradford protein quantization kit. The UV–Visible spectrum of the water solution of sample (1 mg/mL) was measured on a DU-8000 spectrophotometer (Beckman Coulter, Brea, CA, USA) in a wavelength range of 200–800 nm.

2.3.2. Homogeneity and molecular weight evaluation

The homogeneity and molecular weight of polysaccharides were determined by gel permeation chromatography (GPC) in a Waters 515 system coupled with 2410 refractive index detector (Waters, Milford, MA, USA), using a Biosep G4000SWXL column (Tosoh, Japan). The linear regression was calibrated with T-series dextran standards (76.9 kDa, 43.5 kDa, 21.4 kDa and 10.5 kDa) (GE Co., Pittsburgh, PA, USA) and the molecular weights of the polysaccharides were expressed as the dextran equivalent molecular weight.

2.3.3. Monosaccharides composition

The monosaccharides composition of polysaccharides was determined by high-performance liquid chromatography (HPLC) after pre-column derivatization according to the method described by Sun et al. (2009).

Briefly, 1 mg polysaccharide sample was hydrolyzed with 1 mL 30% HCl-methanol at 80 °C for 16 h. The solvent was removed by rotary evaporation at 30 °C. The residue was dissolved in 1 mL 2 mol/L trifluoroacetic acid, and hydrolyzed in a sealed condition at 120 °C for 2 h. Then the solvent was removed by rotary evaporation at 30 °C. The residue was washed with methanol several times to remove the excessive TFA. The residue was finally dissolved in 100 μL ddH_2O for further derivatization. Three repetitions were carried out for each sample.

For the pre-column derivatization, 100 μL of hydrolysate solution or 10 mg/mL monosaccharides standards solution were mixed with 120 μL 0.5 mol/L methanol solution of 1-phenyl-3-methyl-5-pyrazolone and 100 μL 0.3 mol/L NaOH solution, and incubated at 70 °C for 1 h. Then 100 μL 0.3 mol/L HCl solution was added to neutralize the NaOH. Finally, 1 mL dichloromethane was added, and the mixture was vigorously shaken and centrifuged for 5 min. The supernatant, containing the labeled carbohydrates, was filtered through a 0.22 μm membrane and 10 μL of the resulting solution was subjected to HPLC analysis.

The HPLC analysis of labeled monosaccharides was carried out in a Waters 2695-2996 HPLC system (Waters, Milford, MA, USA) coupled with a Sunfire C18 analytical column (4.6 × 250 mm, Waters, USA) operated at a column temperature of 25 °C. The mobile phase consisted of 0.1 mol/L KH_2PO_4 buffer (pH 6.7) (eluent A) and acetonitrile (eluent B). The gradient program was as follows: 0–30 min, 18% of B; 30–60 min, 18–25% of B; 60–65 min, 25–18% of B. The flow rate of the mobile phase was 1 mL/min and the compounds were detected at 245 nm. Sugar identification was by comparison with monosaccharide standards.

2.3.4. Methyl and acetyl esterification detection

The degree of esterification was determined according to the methods of Nergard et al. (2005). The polysaccharide samples (about 2 mg) were hydrolysed with 1 mol/L HCl (0.1 mL) at 100 °C for 2 h before being applied to the GC. The released methanol and acetic acid concentrations were calculated according to their standard curves respectively. 1 mol/L HCl was used as solvent control. Three repetitions were carried out for each sample. The gas chromatography was carried out on an Agilent 6890N (Agilent Technologies Inc., CA, USA) with a flame ionization detector. The column HP-INNOWAX