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Structure characterization of three polysaccharides and a comparative study of their immunomodulatory activities on chicken macrophage

Wentao Fan[a,1], Shijie Zhang[b,1], Pan Hao[a], Pimiao Zheng[a], Jianzhu Liu[c,*], Xiaona Zhao[a,*]

[a] College of Animal Science and Veterinary Medicine, Shandong Agricultural University, Tai'an 271018, PR China
[b] Zhengzhou Ninth People's Hospital, Zhengzhou 450053, PR China
[c] Research Center for Animal Disease Control Engineering Shandong Province, Shandong Agricultural University, Tai'an 271018, PR China

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ABSTRACT

In this study, we evaluated structure characterization and immunomodulatory activity of polysaccharides from *Astragalus aboriginum Richardson* (RAPS), *Atractylodes macrocephala Koidz* (RAMPS) *and Rumia seseloides Hoffm* (RSPS) *in vitro* on chicken macrophage. We found that molecular weight of RAPS and RAMPS was 122.4 and 109.4 kDa higher than 64.71 kDa of RSPS. Glucose occupied 83.95% and 66.39% in RAPS and RAMPS, respectively. RSPS mainly contained glucose and galacturonic acid, which accounted for 32.35% and 29.25%, respectively. The NMR results displayed that RAPS and RAMPS contained β- glucose, β-galactose, and β-galacturonic acid. The backbone was 1 → 6 linked glucose. RSPS showed at least six monosaccharide response signals. *In vitro* experiment, the results showed that RAPS at dosage of 15.62 μg mL^{-1} exhibited significant immunological on chicken macrophage compared to RAMPS and RSPS. Interestingly, costimulatory molecules levels in RSPS group were higher than that of RAPS, which may associated with the special structure of RSPS.

1. Introduction

A biological macromolecule isolated from multifarious plants, epiphytes and animals is known as polysaccharide, possessing a wider spectrum of therapeutic properties, relatively low toxicities and minor side effects, and is largely used as additives in the food and drug industry (Xie, Tang, Jin, Li, & Xie, 2016). Currently, many pharmacologists, chemists and biologists obsess over studying bioactive polysaccharides and the functions such as enhancing the activity of immune function, antivirus, antitumor, fall blood sugar, reducing blood fat have been certified (Poon et al., 2006). RAM (Baizhu) is bitter, sweet and warm in nature, which is conducive to anti-inflammation, anti-ulcer, anticancer, anti-hepatotoxic (Haiyan, Langchong, Meng, & Yalin, 2008). Our previous study showed that RAMPS tp possessed the strong immune-enhancing activity. It would be anticipated as a component of new-type immunopotentiator (Zhao et al., 2016). RA (Huangqi), which is considered the best immune tonic herb in stabilizing and strengthening the protective "Qi" (Zheng et al., 2010). It is also called "the senior of all herbs" in the Essentials of the Materia Medica (A.D. 1694). RS (Fangfeng) is used to expel "wind", relieve exterior syndrome, eliminate dampness, relieve convulsion and diarrhea (Joseph & Susan, 2007). These three medicines indicated good immunoregulating activity based on our lab researches.

In previous study, the active molecules are assumed to have reciprocal effect with some target cells, hence cell-based affinity purification and detect techniques were employed as a screen for bioactive components in medicinal herbs. Macrophage plays an important role in the immune system, and it not only can initiate innate immune responses but also can act as effector cells contributing to the responses process by inflammation, angiogenesis and counteracting infection. For example, *Ganoderma atrum* polysaccharide (PSG-1) was used to work on macrophages of S-180 tumor-bearing mice to probe the PSG-1 perspective of immune regulation (Huang et al., 2015). We aim to find the efficient traditional Chinese medicine as a novel immunostimulant for poultry industry, therefore we choose the chicken macrophages. Considering this factors, a phagocyte cell, chicken macrophage that helps initiate and is involved in all stages of immune responses were cultivated.

Even both clinical and experimental studies had asserted the prophylactic and/or therapeutic efficacy of RAPS, RAMPS, RSPS, but there is still a lack of rigorous scientific evaluation of the three polysaccharides because most previous studies based on only one type of polysaccharide to investigate its immune response, herein we compared three polysaccharides of *Astragalus*

* Corresponding authors.
E-mail addresses: Liujz@sdau.edu.cn (J. Liu), zhaoxn@sdau.edu.cn (X. Zhao).
[1] These authors contributed equally to the work.

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aboriginum Richardson (RAPS), Atractylodes macrocephala Koidz (RAMPS), Rumia seseloides Hoffm (RSPS) to evaluate their immunomodulatory activity. In this research, three different polysaccharides (RAPS, RAMPS, RSPS) were isolated, screened and the effects on cell proliferation, phagocytosis ratio and cytokines release of macrophage were also investigated *in vitro* (Yu, Zhao, Zhu, & Li, 2007).

2. Material and methods

2.1. Materials and chemicals

Astragalus aboriginum Richardson (RA; Inner Mongolia,China), *Atractylodes macrocephala Koidze* (RAM; Zhejiang, China) and *Rumia seseloides Hoffm.* (RS; Hebei, China) were purchased from the YongChun Pharmaceutical Company (Tai an, China).

Dulbecco's phosphate buffered saline (PBS) was from Beijing Solarbio Science & Technology Corporation (Beijing, China). RPMI-1640 medium was from Gibco (New York, USA) and bovine serum albumin was from Invitrogen (Carlsbad, USA). 3-[4,5-Dimethylthiazol-2-yl]-2, 5-diphenyl tetrazolium bromide (MTT) was from AMRESCO (Cleveland, OH, USA). Lipopolysaccharide (LPS), dimethyl sulfoxide (DMSO) was purchased from Sigma (Shanghai, China). Assay kits for Griess (NO), interleukin-1 beta, interleukin-6 (IL-1β, IL-6), tumor necrosis factor-alpha (TNF-α) and interferon beta (IFN-β) were from Shang Hai Lengton Bioscience Co., Ltd. (Shanghai, China). All other chemicals and solvents used were of analytical reagent grade and obtained from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China).

All animals (used in this experiment) sacrificed with anesthesia, handling procedures were performed in strict accordance with the P.R. China Legislation, the Use and Care of Laboratory Animals and with the guidelines established by Institute for Experimental Animals of Shandong Agricultural university and were approved by the College Committee for animal experiment (Permit number: SDAUA-2014-012).

2.2. Extraction and isolation

Polysaccharide was isolated as we previously described (Sun et al., 2015a). Briefly, the RA, RAM and RS samples (500 g) were severally decocted thrice with distilled water (1:10, w/v) and condensed into decoction containing 1 g of materia medica per mL. In order to get high contents, RAPS, RAMPS and RSPS were extracted by one-step ethanol precipitation in which ethanol was added to the decoction to obtain ethanol concentration of 80% (v/v). Three polysaccharides were purified by applying Sevag's method (Huang et al., 2008), then the fractional polysaccharides were passed through a Sephadex A-25 column to remove protein, pigments, and other impurities (Guo, Liang, Lou, & Zhang, 2012). The carbohydrate contents of RAPS, RAMPS and RSPS were 75.68%, 87.21% and 85.27%, respectively, as measured by phenol-sulfuric acid method (Hall, 2013).

2.3. Molecular characterization analysis of RAPS, RAMPS and RSPS

2.3.1. Estimation of apparent molecular mass of RAPS, RAMP and RSPS

The molecular weight distribution of RAPS, RAMPS and RSPS were determined by gel permeation chromatography (GPC) method with a Shodex Ohpak SB-802HO column and a refractive index detector. 20 μL of sample solution (1.0 mg mL^{-1}) was injected each run, with 0.71% sodium sulfate as the mobile phase at a flow rate of 0.5 mL min^{-1} in 35 °C. The linear regression was calibrated with T-series dextran standards (Mw 670, 410, 270, 150, 80, 50, 12, 5, and 1 kDa) (Han et al., 2012).

2.3.2. Monosaccharide composition analysis of RAPS, RAMPS and RSPS

Gas chromatography-mass spectrometry (GC–MS) tests for analysis of the sugar composition were performed on a Agilent 6890 instrument equipped with a DB-5 column (30 m × 0.25 mm × 0.25 μm) and a quadrupole rods mass detector (260 °C). The column temperature was increased from 60 to 260 °C at a rate of 5 °C per min and then kept at 260 °C for 5 min. Split injection (1 μL) was conducted with a split ratio of 1:15 and helium was used as carrier gas with a flow rate of 1.5 mL min^{-1}. The mass spectrometer was operated in electron-impact (EI) mode, the scan range was 50–550 amu, the ionization energy was 70 eV and the scanrate was 0.2 s per scan. The inlet ionization source temperature was 230 °C (Han et al., 2012). The identification and quantification of the monosaccharide of RAPS, RAMPS, RSPS (30 mg, respectively) were achieved by GC–MS analysis. RAPS, RAMPS, RSPS (10 mg, respectively) were hydrolyzed with 2 M trifluoroacetic acid (TFA) at 121 °C for 2 h (Peng et al., 2012). The monosaccharides were analyzed by GC–MS after complete conversion into their acetylated derivation according to the method of Lawrence and Iyengar (1985).

2.3.3. NMR analysis of RAPS, RAMPS and RSPS

RAPS, RAMPS and RSPS (20 mg, respectively) were dried then exchanged with deuterium by lyophilization with D_2O four times. After that, the sample was put in a 5 mm NMR tube dissolved in 1.0 mL of 99.96% D_2O. All spectra were obtained at 98 K on a Bruker Avance 500 MHz NMR spectrometer equipped with a TCI cryoprobe. Tetramethylsilane (TMS) was used as external standard in the ^{13}C NMR test, and D_2O was used as internal standard in the ^{1}H NMR test (Han et al., 2012).

2.4. Macrophage isolation and in vitro drug treatment

Pathogen-free chicken (2 weeks old, 250 g body mass) were obtained from the Experimental Animal Center of the Shandong Agricultural University (Tai'an, China). According to a previously reported methodology (Fan et al., 2015), in brief, after the chicken was killed by exsanguinating from cervical and routinely disinfected, 10 mL sterilized PBS was injected into abdominal cavity in sterile room, abdominal cavities was massaged gently. The lavement was repeated for three times, then combined and centrifuged at 1500 rpm for 15 min, the precipitates of peritoneal macrophages (PMϕ) of chicken were collected, and washed with PBS. The collected cells were seeded and cultured in RPMI-1640 containing 10% heat-inactivated FBS, 100 U mL^{-1} penicillin, and 100 μg mL^{-1} streptomycin at a density of 2×10^6 cells per well. The cells were allowed to adhere for 2 h at 37 °C in a 5% CO_2 incubator. The cultures were then washed twice with PBS, supernatants containing non-wall-adhesive cells were abandoned and the wall-adhesive cells left were PMϕ of chicken. The adherent macrophages were treated with a five-step gradient concentrations 62.5 μg mL^{-1}, 31.25 μg mL^{-1}, 15.625 μg mL^{-1}, 7.813 μg mL^{-1}, 3.906 μg mL^{-1} of the three polysaccharides for 24 h. The control group and LPS group received RPMI-1640 only. Then all cells were added into 20 μL LPS (5 μg mL^{-1}) incubated for 24 h except control group.

2.5. Measurement of immunomodulating activity

2.5.1. Macrophage proliferation measurement

The effect of different polysaccharides extract preparations on PMϕ of chicken proliferation assay was performed by the MTT method (Mosmann, 1983). After LPS incubated for 24 h, 20 μL of