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Characterization and immunostimulating effects on murine peritoneal macrophages of oligosaccharide isolated from *Panax ginseng C.A. Meyer*

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ABSTRACT

Ethnopharmacological relevance: *Panax ginseng C.A. Meyer* has been the most precious and renowned Chinese herb used in Asian countries for the treatment of various medical disorders.
Aim of the study: The aim of this work was to investigate the activation effect on murine peritoneal macrophages of oligosaccharide from the roots of *P. ginseng*.
Materials and methods: In this work, the water-extracted oligosaccharide of *P. ginseng* was (WGOS) isolated and purified from the roots of *P. ginseng* by hot water extraction, ultrafiltration and gel-permeation chromatography. The monosaccharide composition and degree of polymerization (DP) of WGOS were determined by a combination of acid hydrolysis, high performance liquid chromatography (HPLC) and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) analysis. Phagocytosis of macrophages was measured by uptake of the neutral red by macrophages, nitric oxide (NO) was determined by the Griess method, inducible NO synthase (iNOS) activity was determined by colorimetric method using a reagent kit, and tumor necrosis factor-α (TNF-α) was analyzed by enzyme linked immunosorbent assay (ELISA). The reactive species detection kit was used to measure the reactive oxygen species (ROS) level.
Results: WGOS was composed of glucose and the DP was ranging from 2 to 14. Immunological tests showed that treatment of WGOS significantly increased phagocytosis of macrophages, and promoted NO, TNF-α and ROS production. Furthermore, WGOS dose-dependently stimulated NO formation through the up-regulation of iNOS activity.
Conclusions: Taken together, WGOS possessed high immunopotentiating activity and could be developed as a novel immnunostimulant.

1. Introduction

Cancer is a formidable problem for people. It is well known that many anticarcinogens were immunosuppressive agents. They repress tumor growth; meanwhile, they are adverse to immune system of organism. It has become an important aim of research in immunopharmacology and oncotherapy to discover and identify new anti-tumor drugs which can potentialize the immune function (Faanes et al., 1980). In recent years, much effort has been made in the research of natural compounds. And many carbohydrates isolated from various medicinal plants have been described to possess immuno-stimulating activity through their ability to regulate macrophage function (Schepetkin and Quinn, 2006). Macrophages play critical roles in host defense, including phagocytosis, and pathogens and apoptotic cells, production of cytokines, and proteolytic processing and presentation of foreign antigens (Hume, 2006).

Ginseng can refer to either Asian ginseng (*Panax ginseng C.A. Meyer*), American ginseng (*Panax quinquefolius L.*), or Siberian ginseng (*Eleutherococcus senticosus*). All three species of ginseng belong to the Araliaceae family. Based on the grown environments and the cultivated method, ginseng is classified into three kinds, Cultivated Ginseng (CG), Mountain Cultivated Ginseng

Abbreviations: *P. ginseng*, *Panax ginseng C.A. Meyer*; WGOS, Water-extracted oligosaccharide of *P. ginseng*; CWGOS, Crude water-extracted ginseng oligosaccharide; DP, Degree of polymerization; HPLC, High performance liquid chromatography; MALDI-TOF MS, Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; NO, Nitric oxide; iNOS, Inducible NO synthase; ELISA, Enzyme linked immunosorbent assay; TNF-α, Tumor necrosis factor-α; ROS, Reactive oxygen species; DHB, 2, 5-Dihydroxybenzoic acid; MTT, 3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide; LPS, Lipopolysaccharide; ConA, Concanavalin A; PBS, Phosphate-buffered saline; UV, Ultraviolet–visible; DCFH-DA, 2′, 7′-dichlorofluorescein diacetate; DCF, 2′, 7′-dichlorofluorescein; DCFH, Non-fluorescent 2′, 7′-dichlorofluorescein; ROI, Reactive oxygen intermediates

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(MCG) and Mountain Wild Ginseng (MWG). CG is cultivated artificially in farms and contributes the major quantity of ginseng in the current market (Liu et al., 2008). It is usually harvested after 5–6 years of cultivation. MCG, grown in forests and mountains, can be considered to mimic mountain wild ginseng; MWG grows in natural environments in the deep mountains. Normally MCG is harvested after 10–20 years; MWG is rare and expensive, and the exact age is usually not clear.

The root of *P. ginseng* has been used for a long time in Asian countries as a traditional medicine. It has been used commonly among the populace for benefiting Qi, promoting the production of body fluid, calming the nerves, etc. Many active ingredients in *P. ginseng*, including ginsenosides, ginseng peptides and ginseng polysaccharides, have been studied extensively for nutritional properties and medicinal properties (Liu and Xiao, 1992; Gillis, 1997). During the past decades, the bioactivities of the polysaccharides from *P. ginseng* have attracted more and more attention because of their immunomodulatory, antitumor (Ni et al., 2009), antioxidant (Luo and Fang, 2008), antiradiation (Song et al., 2003), antiadhesive (Lee et al., 2006) and hypoglycemic effects (Suzuki and Hikino, 1989). However, little is known about the bioactive properties of oligosaccharides from *P. ginseng*, and essentially nothing is known about their potential immunoregulatory properties. Recently, we have isolated the oligosaccharide from *P. ginseng*, and our preliminary immunopharmacological tests have demonstrated that the oligosaccharide markedly promoted lymphocyte proliferation. The aim of the present work was to evaluate the physico-chemical properties and investigate the possible immunoregulatory effect of *P. ginseng* oligosaccharide on stimulation of macrophages, using an in vitro tissue culture system.

2. Material and methods

2.1. Experimental animals

Male ICR mice (6–8 weeks old) weighing 18–22 g were purchased from Pharmacology Experimental Center of Jilin University and acclimatized for 3 days prior to use. The mice were maintained under controlled conditions with a temperature of 24 ± 1 °C, humidity of $50 \pm 10\%$, and a 12/12-h light–dark cycle (lights on from 6:00 am to 6:00 pm). Rodent laboratory mouse chow and water were provided ad libitum. All animals were maintained and used in strict accordance with the PR China legislation on the use and care of laboratory animals and the guidelines issued by Experimental Animal Center of Changchun University of Chinese Medicine and were approved by the university committee for animal experiments. A total of 120 experimental mice were used in these experiments; 30 mice were used for each experiment and 10 mice each time.

2.2. Materials

The cultivated *P. ginseng* roots used in this experiment were 5 years old, and collected from Changbai Mountain, Jinlin province, China in August 2010, and identified by Professor Shumin Wang at the College of Pharmaceutical Sciences, Changchun University of Chinese Medicine. A voucher specimen is kept at the herbarium of Changchun University of Chinese Medicine (Voucher no. 20100016). Dextran 1000 was purchased from Shanghai Lengton Bioscience Co., Ltd. Sephadex G-25 was purchased from Pharmacia Fine Chemical AB, Uppsala, Sweden. Dimethyl sulfoxide, 2, 5-dihydroxybenzoic acid (DHB), m-hydroxydiphenyl, 3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT), Concanavalin A (ConA) and lipopolysaccharide (LPS) were purchased from Sigma Chemical Co. Mouse TNF-α ELISA kits were purchased from Dakewe Biotech (Beijing, China). RPMI-1640 medium and newborn bovine serum were from Gibio Invitrogen Co. The complete RPMI-1640 medium, used for immunological tests, was supplemented with 10% (v/v) heat-inactivated, endotoxin free newborn bovine serum, penicillin 100 IU/ml and streptomycin 100 μg/ml, pH 7.4. All other reagents were of analytical grade.

2.3. Preparation for the oligosaccharide

To remove ginsengosides, the air-dried *P. ginseng* root (1.0 kg) was crushed and extracted with 95% ethanol (3 L) under reflux extraction for 2 cycles of 2 h each, at 40 °C. The whole extract was filtered through Whatman filter paper. The residue was first dried at room temperature to remove ethanol and then dried at 50 °C for 12 h. The dried residue (500 g) from the plant materials was further extracted with distilled water (10 L) at 70 °C three times, for 90 min each time, and filtered through Whatman filter paper. The aqueous extract was concentrated under a reduced pressure and lyophilized to yield the crude water-extracted ginseng oligosaccharide (named as CWGOS, 260.7 g). CWGOS (100 g) was re-dissolved in 1 L distilled water and dialyzed against distilled water for 72 h in a dialysis sack (molecular weight cut-off of 3000 Da). The fraction out of dialysis sack ($Mw < 3000$ Da) was collected and concentrated at 50 °C in vacuum and lyophilized (yield: 71.1 g). Subsequently, the oligosaccharide was further purified through gel-chromatography, and the appropriate fractions were combined, concentrated and lyophilized to give ginseng oligosaccharide WGOS (Wan et al., 2011).

2.4. Analysis of chemical and monosaccharide composition

Total sugar content was determined by phenol–sulfuric acid method using glucose as standard (Dubois et al., 1956). Uronic acid was determined by the m-hydroxydiphenyl method using galacturonic acid as standard (Tullia et al., 1991). Briefly, WGOS solution (10 mg/ml, 0.4 ml) in water was added to 40 μl of 4 M sulfamic acid–potassium sulfamate and mixed thoroughly. Then the mixture was added to 2.5 ml of concentrated sulfuric acid and heated in a boiling water bath for 20 min. After being restored to room temperature, 40 μl of m-hydroxydiphenyl reagent was added. The absorbance was measured at 525 nm with a 50-probe UV–vis spectrophotometer (VARIAN Ltd., Australia). High performance liquid chromatography (HPLC) was used to determine its sugar composition (Zhang et al., 2009). Briefly, the sample (2 mg) was hydrolyzed with 0.5 ml of 2 M trifluoroacetic acid (TFA) in an ampoule (2 ml). The ampoule was sealed under a nitrogen atmosphere and kept at 120 °C for 1 h, and the excess acid was completely removed by co-distillation with ethanol. After removing the acid, the products of hydrolysis were derivatized with 1-phenyl-3-methyl-5-pyrazolone (PMP) according to the method in the literature (Honda et al., 1989). The monosaccharide derivatives were analyzed by an Agilent RRLC 1200 SL system (Agilent Technologies, Wilmington, USA), equipped with a DIKMA Inertsil ODS-3 column (4.6 i.d. × 150 mm, 5 μm, Dikma, Japan), detected by a UV–vis DAD detector and connected to a Chemstation system. The PMP derivative (20 μl) was injected, eluted with 82.0% phosphate-buffered saline (PBS, 0.1 M, pH 7.0) and 18.0% acetonitrile (v/v) at a flow rate of 1.0 mL/min at room temperature. The wavelength for UV detection was 245 nm.

2.5. Ultraviolet–visible (UV) analysis

In order to investigate whether there were protein and/or nuclear acid in WGOS, the oligosaccharide (0.5 mg) was dissolved in distilled water (5 ml). Then UV–vis absorption spectra were