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# Extraction, characterization of a Ginseng fruits polysaccharide and its immune modulating activities in rats with Lewis lung carcinoma

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ABSTRACT

In this study, one polysaccharide (GFP1), with an average molecular weight of $1.4 \times 10^5$ Da, was isolated from Ginseng fruits. GFP1 was composed of galactose, glucose, rhamnose, and arabinose in a molar ratio of 6.1:2.0:1.1:3.2, and had a backbone mainly consisting of (1 → 6)-linked-Gal*p*, (1 → 3,6)-linked-Gal*p* and (1 → 3,6)-linked-Glc*p* residues, which was terminated with terminal (1 → )-linked-Ara*f* or -Rha*p* attached to O-3 position of (1 → 3,6)-linked-Gal*p* and (1 → 3,6)-linked-Glc*p*. We also evaluated the effect of GFP1 on anti-tumor immune response in Lewis lung carcinoma (LLC)-bearing mouse model and explored the possible mechanism. GPF1 could significantly inhibit tumor growth and lung metastasis in vivo, increase the relative spleen and thymus weight, promote ConA or LPS-induced spleen lymphocytes proliferation, elevate the activities of NK cell in spleen, and increase the serum concentration of interleukin-2 (IL-2) and interferon-γ (IFN-γ), as well as the ratio of $CD4^+/CD8^+$ in LLC-bearing mice. All these findings implied that GFP1 could effectively inhibit tumor growth and lung metastasis via activating immune function and provide insights into the mechanism of GFP1 in the prevention and treatment of lung cancer.

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## 1. Introduction

Cancer is one of the most serious diseases that damage human death in economically developed and developing countries (Jemal et al., 2011). To date, the major approaches for treating cancer consist of surgery, radiation, chemotherapy, and immunotherapy (Gibbs, 2000). Conventional cancer chemotherapy is one of the most frequently used therapeutic modalities for the treatment of cancer, but it generally produces severe side effects due to drug resistance and dose-limiting toxicities, especially through the destruction of lymphoid and bone marrow cells (Li et al., 2010). As a result, many cancer patients die of other secondary diseases, rather than from the cancer itself (Chihara, 1992; Huettemann & Sakka, 2005). Several lines of direct evidence show that many polysaccharides extracted from herbs exhibit chemoprotective and antitumor activities through the immune response of the host organism (Bai et al., 2012; Li et al., 2013; Liu et al., 2011; Yang, Xiao, & Sun, 2013). Hence, the search for new polysaccharide with specific health benefits for immunological activities is of current interest to scientists in cancer research.

*Panax ginseng* C. A. Mey, a well-known traditional herbal medicine, has been used for thousands of years in China and other Asian countries for medicinal purposes, particularly for treatment of cancer, diabetes, and heart problems (Helms, 2004). It also has been reported that the leaves and fruits of *P. ginseng* possess the same pharmacological activity as the roots have (Shin, Kiyohara, Matsumoto, & Yamada, 1997). So they will be available for clinical uses like the roots. There is growing evidence in the literature that Ginseng fruits possess an array of interesting pharmacological actions, such as cardioprotection, vasorelaxant, antistress and neuroprotection. In a series of chemical investigations, a number of principal ingredients, mainly various ginsenosides, were isolated from Ginseng fruits (Wang, Li, Zheng, & Yang, 2004). Several ginsenosides from Ginseng fruits have been reported to have anticancer effects in a broad spectrum of human cancer cells (Wang et al., 2007a,b). However, the anti-tumor activity and immune regulation of purified Ginseng fruits polysaccharide has not yet been well documented in vivo. Therefore, in the present study, we establish an animal tumor model with highly metastatic Lewis lung carcinoma (LLC) tumors in C57BL/6 mice to examine whether Ginseng fruits polysaccharide is involved in the induction of anti-tumor

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immune response in tumor-bearing hosts, and also explore the underlying mechanisms.

## 2. Materials and methods

### 2.1. Plant material

Ginseng fruits were purchased the Medicinal Market of Hebei Anguo (Anguo, China).

### 2.2. Chemicals

3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), cyclophosphamide (CTX), Trifluoroacetic acid (TFA), bovine serum albumin (BSA), dimethyl sulfoxide (DMSO), standard monosaccharides (arabinose, rhamnose, fucose, xylose, galactose, glucose, mannose, glucuronic acid and galacturonic acid) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). DEAE-cellulose-52 and Sepharose CL-6B were purchased from Pharmacia Co. (Uppsala, Sweden). PMI 1640 medium, fetal calf serum (FCS), penicillin and streptomycin (cell culture grade) were purchased from Gibco (Grand Island, NY, USA). FITC-conjugated anti-CD4 and PE-conjugated anti-CD8 were purchased from Becton Dickinson PharMingen (San Diego, CA). Interleukin-2 (IL-2) and interferon-γ (IFN-γ) ELISA kits were from R&D Systems (Minneapolis, MN). Other reagents used were of analytical grade.

### 2.3. Isolation and purification of polysaccharide

The Ginseng fruits (500 g) were defatted twice by soaking in 95% ethanol (10 l) for 5 days at room temperature (25 °C) to remove impurities and small lipophilic molecules, and then extracted with boiling water at 90 °C, each for 5 h. The residue was re-extracted by this procedure for three times. After filtration, the extracts were treated with Sevag reagent to remove proteins, concentrated, and dialyzed against distilled water and running water for 48 h, respectively. The remains were centrifuged, and a fourfold volume of 95% ethanol was added to the supernatant to precipitate the polysaccharides under vigorous stirring. After standing overnight at 4 °C, the precipitate was obtained by centrifugation, concentrated under reduced pressure, and finally lyophilized in vacuum to give the water-extracted crude polysaccharide GFCP (35.4 g, 7.0%).

GFCP was further subjected to size-exclusion and anion-exchange chromatography for the fractionation of this crude polysaccharide. GFCP (35.4 g) was re-dissolved in distilled water, filtered through 0.45 μm filters, centrifuged and then the supernatant was applied to a column (3 cm × 30 cm) of DEAE-cellulose-52, which was eluted successively with distilled water and a gradient of 0 → 2 mol/l NaCl at a flow rate of 2.0 ml/min. The fractions were collected using an automated step-by-step fraction collector, and combined and monitored for total carbohydrate using the phenol–sulfuric acid method. Three main fractions were collected, dialyzed and lyophilized to get white purified polysaccharides, and the obtained polysaccharides were designated as GFCP1, GFCP2 and GFCP3, respectively. Each main peak was further purified by gel permeation chromatography on a Sepharose CL-6B column (2.6 cm × 100 cm), and eluted with 0.15 M NaCl with a flow rate of 0.5 ml/min to yield three completely separated fractions (one neutral polysaccharide: GFP1, 3.7 g, 10.4% of GFCP; GFP2, 0.8 g, 2.3% of GFCP; GFP3, 0.6 g, 1.7% of GFCP). Considering the easy access for GFP1, subsequent physicochemical and biological experiments would be focused on polysaccharide fraction GFP1.

### 2.4. Analysis of purified polysaccharides

#### 2.4.1. General analysis

The carbohydrate content was determined by the phenol–sulfuric acid method, using glucose as a standard (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956). The protein content was estimated by the method of Bradford, using BSA as the standard (Bradford, 1976). Total uronic acid content was determined by photometry with *m*-hydroxybiphenyl at 523 nm (Blumenkrantz & Asboe-Hansen, 1973), using galacturonic acid (GalA) as the standard.

#### 2.4.2. Molecular weight and monosaccharide composition analysis

The homogeneity and molecular weight of polysaccharide was determined by high performance size exclusion chromatography (HPSEC) on a Angilent 1100 liquid chromatography instrument (USA), equipped with a TSK-G3000 PWXL columns (7.8 mm ID × 30.0 cm) and a refractive index detector (RID) detector. The sample (2.0 mg) was dissolved in distilled water (2 ml) and passed through a 0.45 μm filter. The mobile phase was 0.05 mol/l $Na_2SO_4$ and the flow rate was at 0.5 ml/min. A 20 μl sample was injected in each run. The molecular weight was estimated by reference to the calibration curve made from a Dextran T-series standard of known molecular weight (T-2000, T-70, T-40, T-20, and T-10).

The polysaccharide was hydrolyzed with 2 M TFA and converted into alditol-acetates for monosaccharide analysis by gas chromatography (Honda, Suzuki, Kakehi, Honda, & Takai, 1981). The resulting alditol-acetates were analyzed by GC using a Vavian 3400 instrument (Hewlett-Packard, Component, USA), and detected with a flame ionization detector (260 °C). The column temperature was held at 150 °C for 2 min, increased to 215 °C at a rate of 2 °C/min, and sequentially increased to 280 °C at a rate of 6 °C/min for 30 min with $N_2$ as the carrier gas and inositol as the internal standard. Quantification was carried out from the peak area.

#### 2.4.3. Methylation and GC–MS analysis

The polysaccharides were methylated thrice according to Needs and Selvendran (1993). The resulting partially methylated products were hydrolyzed, reduced and acetylated as described by Sweet, Shaprio, and Albersheim (1975), and analyzed by gas chromatography–mass spectrometry (GC–MS). The partially methylated alditol acetates were identified by their relative retention times on GC and fragment ions in EI-MS, and the molar ratios were estimated from the peak areas and response factors.

### 2.5. Cell culture

Mouse LLC cells were obtained from KeyGEN Biotech (Nanjing, China) and were cultured at 37 °C under 5% $CO_2$ in a RPMI-1640 medium containing 10% FCS, 100 U/ml penicillin, and 100 μg/ml streptomycin. LLC cells generated an 80–90% confluent layer and were prepared to suspension for animal experiment.

### 2.6. Cytotoxicity assay

Cell viability was determined using the MTT assay. LLC cells were seeded in 96-well plates at a plating density of $1 \times 10^4$ cells/well. After 24 h, cells were exposed to GFP1 at various doses (0, 25, 50 and 100 mg/ml) in fresh RPMI 1640 medium for 48 h. Then 10 μl of MTT (5 mg/ml in PBS) was added to each well and incubated for other 4 h at 37 °C, followed by addition of DMSO (100 μl/well) to each well. The color intensity of samples was measured with an ELISA microplate reader (Thermo Molecular Devices Co., Union City,

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