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Purification, antitumor and immunomodulatory activity of polysaccharides from soybean residue fermented with *Morchella esculenta*

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ABSTRACT

Crude polysaccharides (MPS) from soybean residue fermented with *Morchella esculenta* were extracted and purified by DEAE Sephadex A-50 chromatography and Sephadex G-100 size-exclusion chromatography in sequence. Three main fractions MP-1, MP-3 and MP-4 were obtained during the purification steps. The recovery rates based on MPS used were 26.2%, 29.1% and 18.7% for MP-1, MP-3 and MP-4 respectively. The monosaccharide composition, ultraviolet spectrum, infrared spectrum and NMR of the three fractions were analyzed. Furthermore, the influence of polysaccharides fractions upon activation of macrophage cells (RAW 264.7), antitumor activities of the human hepatocellular cell line (HepG-2) and human cervical carcinoma cells (Hela) *in vitro* were evaluated. The results indicated that the proliferation of MP-3 on RAW 264.7 was 313.57% at 25 μg/mL, which is high while MP-1 had a higher growth inhibition effect on HepG-2 cells of 68.01% at concentration of 50 μg/mL. The fractions of MP-1, MP-3 and MP-4 induced apoptosis in HepG-2 cells and Hela cells by arresting cell cycle progression at the G_0/G_1 phase. These findings suggest that the purified polysaccharides fractions may be a potent candidate for human hepatocellular and cervical carcinoma treatment and prevention in functional foods and pharmacological fields.

1. Introduction

Cancer is one of the most serious diseases that affect human health. It is predicted that almost 21.4 million new cancer cases and over 13.2 million deaths are likely to occur by 2030 [1]. Therefore it is necessary to work on antitumor agents that have low toxic effects and high success against tumor growth.

Polysaccharides isolated from plants, fungi, yeasts and algae have been increasingly utilized in biochemistry and pharmacology. They exhibit a wide range of biological functions, such as anti-inflammatory, antioxidant, antitumor, immunostimulatory, antitumor activity and so on [2–4]. It is worth mentioning that the immunostimulatory and antitumor effects of polysaccharides have attracted considerable attention in the biomedical field for their defense against tumors and for increasing the human body's immunity [5].

Morchella esculenta (M. esculenta) is appreciated not only for its flavor but also for its medicinal and nutritional properties. As previously reported, the crude polysaccharides isolated from soybean residue (SR) fermented with *M. esculenta* have antioxidant activity [6]. However, few examples of polysaccharides isolated from SR fermented with *M. esculenta* have been reported, let alone the purification, characterization, immunomodulatory and antitumor activities of the polysaccharides. To address these shortcomings, this study reports on the purification and characterization of polysaccharides from SR fermented with *M. esculenta*. Their immunomodulatory and antitumor activities were also studied.

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2. Material and method

2.1. Chemicals and reagents

Rhamnose, glucose, mannose, galactose, arabinose, xylose, fructose, propidium iodide (PI), EDTA and Triton X-100, Minimum Essential Medium Eagle (MEM) medium, fetal bovine serum (FBS) and penicillin-streptomycin solution were purchased from Sigma Aldrich, Inc. (Saint Louis, MO, USA). A cell Counting Kit-8 (CCK-8) was purchased from Dojindo Molecular Technologies, Inc. (Kumamoto, Japan).

2.2. Preparation of polysaccharides

The crude polysaccharides were extracted from soybean residue fermented with *Morchella esculenta* following previous work. Briefly, fermented SR was ground to powder and passed through a NO. 60 mesh after drying. The powder was refluxed in 80% ethanol for 6 h to remove monosaccharides and colored materials. Then the residue was washed with 95% ethanol, anhydrous ethyl alcohol, acetone and diethyl ether respectively. Subsequently, extraction was carried out using boiling water for 2 h and the syrup was centrifuged at 7500 × *g* for 15 min. The protein in the concentrated solution was removed by Sevag reagent (chloroform and *n*-butanol in 4:1 ratio) [7]. The extract was dialyzed by deionized water for 72 h and polysaccharides (MPS) were obtained through ethanol precipitation.

100 mg of MPS was redissolved in 5 mL distilled water, filtered through Whatman filter and applied to a DEAE Sephadex A-50 column (2.5 × 40 cm) equilibrated with distilled water. The polysaccharides were fractionated and eluted with distilled water and different concentrations of stepwise NaCl solution (0, 0.05, 0.10 and 0.50 M NaCl) at a flow rate of 2.0 mL/min. The elutes were concentrated to obtain the main fractions, which were then purified by size-exclusion chromatography Sephadex G-100 gel permeation column (2.5 × 40 cm) eluted with 0.1 M NaCl at a flow rate of 0.5 mL/min. The obtained fractions were combined according to the total carbohydrate content quantified by the phenol-sulfuric acid method [8]. The relevant fractions were collected, concentrated, dialyzed and lyophilized. For analysis of biological activity, the polysaccharides fractions were diluted in distilled ultrapure water and filtered through sterile 0.45 μm filters.

2.3. Spectroscopy analysis

Polysaccharides solution (0.2 mg/mL, dissolved in ultrapure water) was scanned on a spectrophotometer UV-1800 (Shimadzu, Tokyo, Japan) from 200 nm to 400 nm. The FTIR spectra (4000–400 cm^{-1}) of the polysaccharides fractions were determined by a Jasco FTIR 3000 spectrophotometer (Jasco, Wakayama, Japan).

The ^{1}H and ^{13}C NMR spectra were performed at 60 °C with a Bruker AVANCE 500 NMR spectrometer (Brucker Co., Billerica, MA, USA). The sample was exchanged with deuterium by freeze-drying in 99% D_2O three times and then deuterium-exchanged polysaccharide (30 mg) was dissolved in 0.5 mL of 99% D_2O.

2.4. Monosaccharide composition analysis

The purified fractions were hydrolyzed according to the method of Shi et al. [9]. After hydrolyzation, approximately 0.1 mL of clear supernatant was added to the auto sampler vials with inserts for injection into the gas chromatograph on a GCMS-QP2010 Plus (Shimadzu, Japan) instrument equipped with a hydrogen flame ionization detector, using a DB-5 column (30 m × 0.25 mm × 0.25 mm). The chromatographic conditions utilized: high-purity helium as the carrier gas with a flow rate of 1 mL/min. The injector and detector temperature were set at 250 °C. The initial column temperature was held at 60 °C for 2 min followed by 15 °C/min to 180 °C and then 4 °C/min to 250 °C for 3 min [10]. Injections were made in the split less mode. The ion source temperature was 250 °C. A 1 μL sample was injected into the column with a split ratio of 10:1. Monosaccharide peaks in the MPS were identified by comparison of retention time and mass spectral fragmentation patterns for monosaccharide standards. The amount of individual monosaccharide was calculated by comparison of the peak areas to the peak area of inositol.

2.5. Cell line and cell culture

Raw murine macrophage cell line RAW 264.7, human hepatocarcinoma cell line HepG-2 and human cervical cancer Hela were obtained from RIKEN Bioresource Center (Tsukuba, Japan). RAW 264.7 macrophages and Hela were grown in MEM medium containing 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. HepG-2 cells were grown in DMEM medium containing 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. Culture was maintained at 37 °C in a humidified atmosphere with 5% CO_2 (ESPEC CO_2 Incubator).

2.6. Immunomodulatory activity

2.6.1. Measurement of cell proliferation ability

The effect of polysaccharides on RAW 264.7 proliferation was estimated by Cell Counting Kit-8 (CCK-8). RAW 264.7 cells were cultured in a 96-well plate at a density of 5×10^4 cells/mL at 37 °C in a humidified atmosphere with 5% CO_2 for 24 h. Then cells were incubated with different concentrations of extracts (0, 6.25, 12.5, 25, 50 and 100 μg/mL) at 37 °C for 24 h. 10 μL of CCK-8 reagent solution was added and incubated at 37 °C for 4 h. The cell viability was determined based on a wavelength of 450 nm using a microplate reader (BIO-RAD Model 550, Japan). Cell proliferation was calculated using the following equation:

$$\text{Cell proliferation rate } (\%) = (A_{sample} - A_{blank1}) / (A_{control} - A_{blank2}) \times 100$$

A_{sample}, optical density value of tested samples with cells, A_{blank1}, optical density value of samples with medium, $A_{control}$, optical density value of control with cells, A_{blank2}, optical density value of control with medium.

2.6.2. Nitric oxide assay

The macrophage cells (1×10^5 cells/mL) were placed into a 96-well plate for 24 h. Then the cells were stimulated with Lipopolysaccharide (LPS) 1 μg/mL and various concentrations of polysaccharides (0, 6.25, 12.5, 25, 50 and 100 μg/mL) for 24 h. After incubation, the nitrite concentration in the culture medium was measured as an indicator of NO production based on the Griess reaction. 50 μL of supernatant was mixed with the same volume of Griess reagent and the resultant mixture was then incubated for 10 min. The absorbance at 570 nm was then measured and $NaNO_2$ was used as the standard to calculate the nitrite concentrations.

2.6.3. Phagocytosis assay

The phagocytic ability of macrophage RAW 264.7 cells was measured with neutral red uptake. The macrophage cells (5×10^4 cells/mL) were cultured in the presence of various concentrations of polysaccharides and LPS (1 μg/mL) in a 96-well plate in a total volume of 100 μL per well for 48 h at 37 °C. 100 μL/well of 0.075% neutral red was added and incubated for 1 h. The culture media was discarded and the macrophages were washed twice with PBS (pH: 7.2-7.4). Then 100 μL/well of the cell lysing solution (50%