Carbohydrate Polymers 118 (2015) 101–106

Contents lists available at ScienceDirect

Carbohydrate Polymers

journal homepage: www.elsevier.com/locate/carbpol

Structure characterization and antitumor activity of a polysaccharide from the alkaline extract of king oyster mushroom

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ARTICLE INFO

Article history:
Received 7 January 2013
Received in revised form 17 October 2014
Accepted 22 October 2014
Available online 30 October 2014

Keywords:
King oyster mushroom
Polysaccharide
Immunomodulatory
Antitumor

ABSTRACT

A water-soluble polysaccharide, designated as KOMAP, was isolated and purified from the alkaline extract of king oyster mushroom, which was composed of glucose (Glc), mannose (Man) and arabinose (Ara) in a molar ratio of 6.2:2.1:2.0. It had an average molecular weight of 2.1×10^4 Da. GC–MS analysis revealed that KOMAP was a linear structure of the polymer with a backbone composed of β-1,4-linked glucopyranosyl and β-1,3,6-linked mannopyranosyl units, which was terminated with α-1-linked arabinofuranosyl unit at C-6 position of β-1,3,6-linked mannopyranosyl residue along the main chain in the ratio of 3.1:1. The results in the animal experiment showed that 50, 100 and 200 mg/mL of KOMAP not only inhibited the tumor growth, but also increased relative thymus and spleen indices, LPS- or ConA-induced lymphocytes proliferation, and serum cytokine IL-2, TNF-α, and IFN-γ levels, as well as the activities of NK cells and CTLs in spleen of Renca tumor-bearing mice. In summary, our data indicate that the KOMAP exerts effective immunoregulatory and anti-tumor activities in vivo.

1. Introduction

During the past decades, polysaccharides and polysaccharide–protein complexes derived from natural sources, such as Traditional Chinese Medicine (TCM) and some medicinal mushrooms, have received much attention as a source of therapeutic agents for the prevention and cure of neoplastic diseases due to their immunomodulatory and antitumor effects (Mitchell, 2003). Several lines of direct evidence showed that the enhancement of host immune defense system by bioactive polymers has been suggested to be a safe means of inhibiting tumor growth without harming the host in contrast to existing cancer chemotherapy, radiotherapy and the majority of anti-tumor chemical compounds (Wang, Sun, Wu, Yang, & Tan, 2014; Yuan, Song, Li, Li, & Dai, 2006). These immunomodulating polysaccharides are regarded as biological response modifier (BRM) i.e. they cause no harm and place no additional stress on the body, but help the body to adopt to environmental and biological stress (Mizuno et al., 2000). From this standpoint, extensive study should be undertaken on polysaccharides isolated from natural sources with improving immunity potential.

Edible mushrooms contain physiologically beneficial bioactive compounds and can be good sources for the cancer treatment (Li et al., 2008; Sarangi, Ghosh, Bhutia, Mallick, & Maiti, 2006, Stajić, Vukojević, & Duletić-Lausević, 2009). As a type of edible mushroom, *Pleurotus eryngii*, commonly called the king oyster mushroom due to its good taste and nutritional value, has rapidly become a highly valued species among consumers in North America, Europe, and Asia (Royse, 1995). This mushroom contains many biologically active substances such as polysaccharides, lipids, peptide, sterols, and dietary fiber (Liu et al., 2010). Antitumor activity of the crude polysaccharides or purified polysaccharides from the water extract of king oyster mushroom have already been carried out by several works (Kim et al., 2004; Zhang, Cheung, Zhang, Chiu, & Ooi, 2004; Zhang, Zhang, Cheung, & Ooi, 2004; Yang et al., 2013). However, the chemical components and mechanisms of anti-tumor activity of purified polysaccharides from the alkaline extract of king oyster mushroom remain unclear. Therefore, the present paper was

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http://dx.doi.org/10.1016/j.carbpol.2014.10.058
0144-8617/

concerned with the isolation, chemical characterization and evaluation of the anti-tumor activity of the polysaccharide KOMAP from the alkaline extract of king oyster mushroom.

2. Materials and methods

2.1. Materials and chemicals

King oyster mushroom was brought from one of the local markets in Zhengzhou city of China. DEAE-cellulose and Sephadex G-200 were purchased from Pharmacia Chemical Co. 3-(4, 5-dimethyl-2-thiazol)-2,5-diphenyl-2H-tetrazolium bromide (MTT); 1-phenyl-3-5-pyrazolone (PMP), Concanavalin A (Con A), lipopolysaccharide (LPS), T-series Dextran (T-2000, T-70, T-40, T-20, and T-10), the monosaccharide standards, trifluoroacetic acid (TFA) and bovine serum albumin (BSA) were purchased from Sigma Co. (St. Louis, MO, USA). Fetal bovine serum (FBS) and RPMI-1640 medium were purchased from Gibco Invitrogen Co. (Grand Island, NY, USA). Enzyme-linked immunosorbent assays (ELISA) for mouse interleukin-2 (IL-2), tumor necrosis factor-α (TNF-α) and interferon-γ (IFN-γ) were purchased from Pierce Biotechnology (Rockford, IL, USA). Commercial reagent kits for aspartate aminotransferace (AST), alanine aminotransferace (ALT), blood urea nitrogen (BUN), uric acid (UA) and creatinine (CRE) were purchased from Lanji Technology Development Co. (Shanghai, China). All the other chemicals used were of analytical grade.

2.2. Extraction, isolation and purification of polysaccharides

The dried king oyster mushroom (500 g) was ground to powders, and submitted to remove lipids by extracting 3 times with 10 vol of 95% EtOH under reflux at 75 °C for 3 h each time. The residue obtained was then extracted thrice with 15 vol of boiling water under reflux until no reaction of phenol–sulfuric acid and then dipped into 5% alkali solution for 24 h for three times. The extract was filtered through gauze and the suspension was then neutralized with hydrochloric acid (0.1 M) and concentrated to one-tenth of the volume with a rotary evaporator at 80 °C under vacuum. The proteins in extract were removed from the extract by using Sevag reagent (Staub, 1965). After the Sevag reagent was removed, the mixture was further precipitated with 4 vol of 95% EtOH at 4 °C for 24 h. After centrifugation, the resulting precipitate (crude polysaccharides, KOMCP) was washed sequentially with ethanol, acetone, ether, and vacuum-dried.

The crude polysaccharide KOMCP was applied to a DEAE-cellulose column (2.0 cm × 40 cm), eluted stepwise with distilled water, 0.5 and 1.0 M NaCl, respectively. The eluate (6 ml) was collected and monitored for carbohydrate content using phenol–sulfuric acid method at 490 nm (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956). According to the elution profile, the eluates were combined, concentrated, dialyzed and lyophilized to give three fractions. The fraction containing polysaccharide eluted by distilled water solution was further applied to a column (2.5 cm × 100 cm) of Sephadex G-200 equilibrated with 0.1 M NaCl at a flow rate of 60 ml/h. After collection, the relevant fractions were concentrated, dialyzed and lyophilized to produce white power, of which the KOMAP was used in the subsequent studies.

2.3. General analysis of the polysaccharide

The total neutral sugar content and total uronic acid content use Glc and GalA as standard, respectively. The former was determined by the reaction with phenol in the presence of sulfuric acid at 486 nm (Dubois et al., 1956); while the latter was determined by photometry with m-hydroxybiphenyl at 523 nm (Blumenkrantz & Asboe-Hansen, 1973). Proteins were estimated by the Bradford assay (Bradford, 1976), using BSA as the standard.

The average molecular weight was determined by the High-performance gel-permeation chromatography (HPGPC), which was performed on a Waters HPLC system (Allances 2695, Waters, USA) with a column of TSK-GEL G3000 SWXL (7.8 mm × 300 mm), and a Waters 2410 differential refractometer. The mobile phase was 0.1 mol/l $NaNO_3$, and the flow rate was 1 ml/min. The sample (2 mg) was dissolved in the mobile phase (0.2 ml) and centrifuged. 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).

Polysaccharide samples (2 mg) were first methanolyzed with anhydrous methanol containing 2 M HCl at 80 °C for 16 h and then hydrolyzed with 2 M TFA at 120 °C for 1 h. The resulting hydrolysates were derivatized with PMP according to the method reported by Yang et al. (Yang, Zhao, Wang, Wang, & Mei, 2005) and analyzed on a DIKMA Inertsil ODS-3 column (4.6 mm × 150 mm) connected to a Shimadzu HPLC system (LC-10ATvp pump and SPD-10AVD UV-VIS detector) with UV absorbance detection at 245 nm. 20 μL of the PMP derivative was injected for each run and eluted with 80.0% PBS (0.1 M, pH 7.0) and 20.0% acetonitrile (v/v) at a flow rate of 1.0 mL/min.

2.4. Methylation analysis

The polysaccharide, KOMAP (20 mg), was methylated using according to Needs and Selvendran (1993). Complete methylation was confirmed by the disappearance of the OH band (3200–3700 cm^{-1}) in the IR spectrum. After completed methylation, the methylated products were hydrolyzed, then reduced and acetylated as described by Sweet, Shapiro, and Albersheim (1975). The partially methylated alditol acetates were then analyzed on a gas chromatography/mass (GC/MS) spectrometer (QP 2010, Shimadzu, Kyoto, Japan) with a SP-2330 capillary column (30 m × 0.25 mm, 0.2 mm film thickness) to analyze the glycosidic linkage. The temperature program was set as follows: the initial temperature of column was 160 °C, increased to 210 °C at 5 °C/min, held for 5 min at 210 °C, and then 210–250 °C at 5 °C/min.

2.5. Animals preparation and experiment design

Female specific pathogen-free (SPF) BALB/c mice (weighing from 18 to 20 grams) acquired from Experimental Animal Center, the Zhengzhou University (Zhengzhou, China), were kept for a minimum of one week prior to the study so that they were acclimatized. They were also free access to standard food and water and maintained in a SPF room with air-conditioning providing a 12/12 h light/dark cycle. Temperature inside the room was kept at 24 ± 1 °C, and humidity at 50 ± 10%. Animals were handled according to the rules and regulations of Institutional Animal Ethics Committee (IAEC) of Zhengzhou University.

The diluted Renca tumor cell suspension (2×10^5 cells) was subcutaneously injected into the armpit for 0.2 ml per mouse to prepare tumor-bearing mice. A total of 40 mice were allocated in random into four groups (10 mice in each group) 24 h after the tumor inoculation and treated with 50, 100 and 200 mg/kg of KOMAP for 10 consecutive days once daily by gavage, and another 10 tumor-bearing mice were administrated with sterile physiological saline intragastrically once daily (10 m/kg) served as normal control. One hour after the last drug administration, the rats were weighed and sacrificed by cervical dislocation. The tumor mass and their spleens and thymuses were instantly dissected, weighed and calculated according to the following formulae: Organ index (mg/10 g) = organ weight/body weight; the inhibitory rate