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RESEARCH ARTICLE

Chemical Characterization and Antitumor Activity of an Exopolysaccharide from *Pholiota Squarrosa* Quel. AS 5.245

Haizhen Zhao, Juan Wang, Fengxia Lv, Xiaomei Bie, and Zhaoxin Lu

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Abstract A water-soluble exopolysaccharide (PESP-1) was extracted and purified using DEAE Sephadex A-50 and Sephadex G-100 columns from a submerged culture broth of *Pholiota squarrosa* Quel. AS 5.245. PEPS-1 was investigated for antitumor activity against Heps tumors implanted in mice. An inhibition rate of 78.46% at a dosage of 100 mg/kg was observed. PEPS-1 significantly ($p<0.05$) increased the relative spleen/thymus indices of Heps tumor-bearing mice at a dosage of 100 mg/kg, compared with controls. Antitumor properties were probably related to stimulation of the immune response. Preliminary physicochemical analysis identified PEPS-1 as a heteropolysaccharide mainly containing D-mannose, D-glucose and D-galactose at molar ratios of 50:33:18. Small amounts of D-rhamnose and D-xylose were also detected. The average Mw of PEPS-1 was 3.26×10^4 Da. Structural features probably played an important role in the antitumor activity of PEPS-1.

Keywords: exopolysaccharide, *Pholiota squarrosa*, antitumor activity

Introduction

The World Health Organization estimated that more than 7 million people died of cancer in 2012 and new cancer cases will rise from an estimated 14 million annually in 2012 to 22 million within the next two decades (1). Today, development of anti-cancer drugs is a worldwide issue. Screening of new anti-cancer drugs from natural products has become a focus of anti-cancer drug development.

Polysaccharides are the most diverse family of biopolymers. Many polysaccharides have wide applications. In recent years, much attention has been focused on polysaccharides because of multiple bioactivities and pharmacological actions. Polysaccharides have been isolated from plants, fungi, yeasts, mushrooms, and sea organisms using extraction (2-4). A wide range of bioactive polysaccharides isolated from edible fungi of the genera *Ganoderma, Agaricus,* and *Lentinus,* have been widely investigated (5,6), and have shown interesting biological properties, such as anti-inflammatory, immunomodulating and antitumor activities (7-9).

Edible mushrooms of the genus *Pholiota* Kummer (Family Strophariaceae, Order Agaricales, Class Basidiomycetes) have also been studied. *Pholiota* Kummer is a genus of mushrooms rich in vitamins, amino acids, trace elements, lipids, and polysaccharides (10), and many varieties exhibit pharmacologic activities, including *Pholiota nameko, P. aurivella, P. aiposa, P. flammans,* and *P. lubrica* (11). The brown fungus *P. squarrosa* is fairly widely distributed in America, Europe, and China. Information is lacking regarding isolation, purification, and activity determination of polysaccharides from *P. squarrosa*. In this study, strain *P. squarrosa* (Pers. *ex* Fr.) Quel. AS 5.245 was cultured, and an exopolysaccharide was purified from the culture broth using DEAE-50 cellulose and Sephadex G-100 columns chromatography. Chemical properties and antitumor activities of the exopolysaccharide were characterized *in vivo*.

Materials and Methods

Materials and chemicals *P. squarrosa* (Pers. *ex* Fr.) Quel. AS 5.245, obtained from The China Center for Type Culture Collection (Wuhan, China) was grown on potato

Haizhen Zhao, Juan Wang, Fengxia Lv, Xiaomei Bie, Zhaoxin Lu (✉)
College of Food Science and Technology, Nanjing Agricultural University, Nanjing 210095, China
Tel/Fax: +86-25-84396583
E-mail: fmb@niau.edu.cn

dextrose agar (PDA) slants at 4ºC, and sub-cultured every 3 months. DEAE Sephadex A-50 and Sephadex G-100 were purchased from Pharmacia Biotech (Tokyo, Japan). Hydroxylamine hydrochloride, $BaCO_3$, and concentrated sulfuric acid were purchased from Fluka (St. Gallen, Switzerland). D-Mannose, D-glucose, D-galactose, D-arabinose, D-rhamnose, D-xylose, and inositol were purchased from Sigma Chemical Co. (St. Louis, MO, USA). All other reagents were of chromatography or analytic grade.

Production, isolation, and purification of an exopolysaccharide (EPS) Fungal cells were cultured at 28ºC and 140 rpm using a HYG-A cabinet shaker (Taicang, Jiangsu, China) for 8 days in potato dextrose media without agar. After incubation, cells were removed from the culture medium using a ceramic membrane (Jiangsu Jiuwu Hi-Tech Co. Ltd., Nanjing, China). The filtrate was precipitated using a 4× volume of 95% (v/v) cold ethanol and kept at 4ºC for 20 h. The exopolysaccharide was collected using a DL-5 centrifuge (Shanghai Anting Scientific Instrument Factory, Shanghai, China) at 8,000×*g* for 10 min, then dissolved in distilled water. The solution was deproteined following the Sevage method (12), followed by exhaustive (5 days) dialysis using distilled water. Finally, the concentrated supernatant was lyophilized using an Alpha 1-2 vacuum freezing dryer (Martin Christ Gefriertrocknungsanlagen GmbH, Osterode am Harz, Germany) to obtain crude, brown EPS.

Crude EPS was dissolved in distilled water and then was fractionated using a DEAE Sephadex A-50 chromatography column (2.5×30 cm). The column was eluted with distilled water at a flow rate of 0.5 mL/min with monitoring following the phenol-sulfuric acid method (13). The collected fraction was further fractionated using a Sephadex G-100 column (2.8 cm×80 cm), and eluted with distilled water at a flow rate of 0.2 mL/min. All fractions were collected using a BSZ-100 automated fraction collector (Shanghai Qingpu-Huxi Instruments Factory, Shanghai, China) and monitored following the phenol-sulfuric acid method. Finally, collected fractions were lyophilized using an Alpha 1-2 vacuum freezing dryer (Martin Christ Gefriertrocknungsanlagen GmbH) to obtain a purified white power, named PEPS-1, which was subjected to subsequent analysis.

Chemical characterization of PESP-1

***Ultraviolet assay*:** PEPS-1 was re-dissolved in distilled water and subjected to scanning from 190 nm to 400 nm using a UV-2450 spectrophotometer (Shimadzu, Tokyo, Japan).

***Homogeneity and molecular weight determination*:** The homogeneity and average molecular weight (Mw) of PEPS-1 were determined using gel-permeation chromatography (GPC) in combination with Waters 600 HPLC (Waters Co., Milford, MA, USA). A sample dissolved in 0.1 mol/L $NaNO_3$ was passed through a 0.45 μm filter and applied to a Ultrahydrogel linear column (7.8×300 mm) and eluted with 0.1 mol/L $NaNO_3$ at a flow rate of 0.9 mL/min. Detection used a differential refractive index detector (RID) (Waters 2410; Waters Co.). Dextran standards (M_W 4.1, 71.4, 110, 200, and 580 kDa) were injected, then the resulting elution time was plotted against the logarithm of the Mw value using GPC software.

***Analysis of the monosaccharide composition*:** PEPS-1 was hydrolyzed using a polysaccharide 5 mL of 1.0 mol/L H_2SO_4 at 100°C for 10 h in screw-capped glass tube. After removal of residual acid using $BaCO_3$, hydrolysates were filtered and dried under vacuum with successive reduction using hydroxylamine hydrochloride and acetylation with Ac_2O at 90°C for 30 min. The resulting derivatives of saccharin acetyl were extracted using chloroform and analyzed using GC performed on a SP2000 instrument (Lunan-Ruihong, Shandong, China) equipped with an HP-1 capillary column (Lunan-Ruihong, Shandong, China) (30 m×320 μm×0.25 μm) and detected using a flame ionization detector (250°C). The injector temperature was kept at 260°C. D-Mannose, D-glucose, D-galactose, D-arabinose, D-rhamnose, D-xylose and inositol neutral monosaccharides were used as references.

***In vivo* antitumor activity against Heps tumor** Kunming mice between 6 and 8 weeks old (18-22 g) were purchased from the Experimental Animal Center of Qinglong Mountain (Nanjing, Jiangsu Province, China). Mice were housed under normal laboratory conditions of a temperature of 22±2°C, a 12/12 h light-dark cycle, and free access to standard rodent chow and water. All experimental procedures were in compliance with the National Institutes of Health Guide for Care and Use of Laboratory Animals (14).

Under sterile conditions, 0.2 mL of mouse ascites (including 1×10^8 Heps cells/mL) were injected into the right axilla of Kunming mice on day 0. On the second day, mice were randomly subdivided into 4 groups of 10 each. Mice in model Group A were treated with physiological saline at a dosage of 25 mL/kg. As a positive control, mice in Group B were intraperitoneally injected with 5-fluorouracil (5-Fu) at a dosage of 25 mg/kg. Groups C and D mice were intraperitoneally injected with PEPS-1 at 50 and 100 mg/kg, respectively. Subsequently, mice were treated daily for 8 days. Then, 24 h after the last drug administration, animals were euthanized using CO_2 treatment for 5-7 min. Tumor, thymus, and spleen tissues of mice from all groups were excised and weighed using an AUW120D microbalance (Shimadzu).

The antitumor activities of tested tissue samples were expressed as a percentage calculated as $[(A - B)/A] \times 100\%$, where A and B are average tumor weights of control and

treatment group mice, respectively. The spleen index was expressed as the weight of the spleen (mg)/body weight (g). The thymus index was determined using the same method. The body weight ratio was calculated as the average percentage of the final body weight to the initial body weight.

Statistical analysis All results were expressed as a mean±standard deviation (SD). Data were analyzed using the standard *t*-test function of the SAS software package (SAS Institute, Cary, NC, USA). Statistical significance was defined as $p<0.05$.

Results and Discussion

Isolation and purification of the water-soluble exopolysaccharide fraction Crude EPS was obtained from the culture broth of *P. squarrosa* (Pers. *ex* Fr.) Quel. AS 5.245 using ethanol precipitation, deproteinization, and lyophilization. The crude EPS yield was 11.5%. Crude EPS was fractionated using a DEAE-50 cellulose column to obtain a single fraction (Fig. 1), which was collected via monitoring the carbohydrate content using a phenol-sulfuric acid assay at 490 nm. EPS was a neutral polysaccharide as it was eluted using water. EPS was subjected to gel filtration on a Sephadex G-100 column, resulting in a purified white polysaccharide named PEPS-1 (Fig. 2).

Characterization of PEPS-1 An ultraviolet assay showed that PEPS-1 exhibited no absorption at 280 or 260 nm in the UV spectrum (Fig. 3), indicating an absence of proteins and nucleic acids (15). The Mw of PEPS-1 was calculated based on HPLC-GPC. PEPS-1 was analyzed based on the HPLC spectrum and the retention time for PEPS-1 was determined. The Mw was calculated using the standard curve eq.:

$$\text{Log Mw} = 13.1 - 0.474\text{T} \ (r^2 = 0.9987)$$

where Mw is the Mw value of standard dextrans and T is retention time (min). Based on the HPLC spectrum, the retention time of PEPS-1 was 19.733 min. The average Mw of PEPS-1 was calculated as 3.26×10^4 Da substituting 19.733 min into eq. The Mw value obtained was smaller than the value for EPS isolated from *P. nameko* with an average Mw of 1.14×10^5 Da (16).

Analysis of the sugar composition of PEPS-1 indicated the presence of D-mannose, D-glucose, and D-galactose at molar ratios of 50:33:18, which accounted for the majority of monosaccharides present. Small amounts of D-rhamnose and D-xylose were also detected (Fig. 4). Polysaccharides may exist in linear or branched forms. According to the

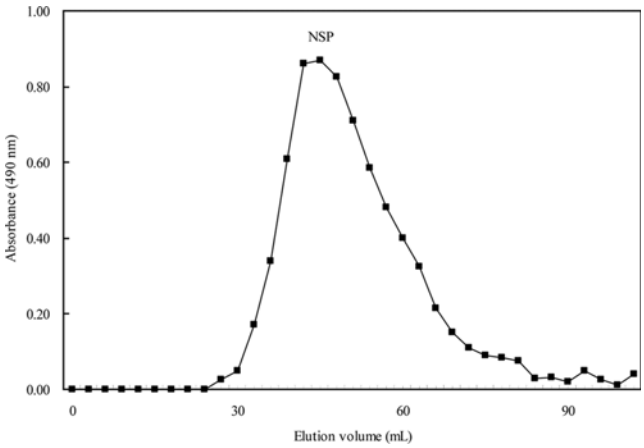

Fig. 1. Anion exchange chromatograph of the *Pholiota squarrosa* Quel. AS 5.245 exopolysaccharide on a DEAE Sephadex A-50 column.

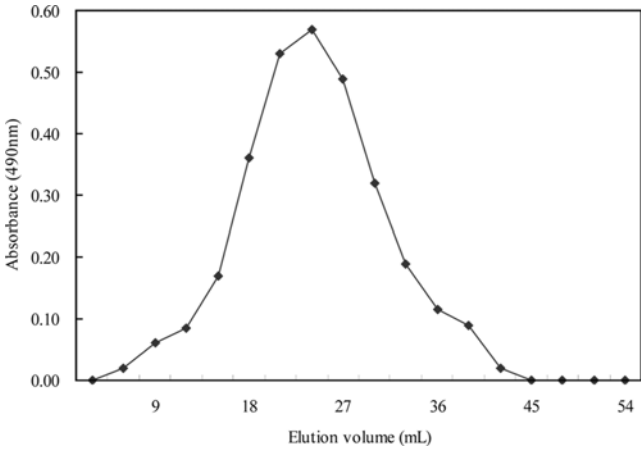

Fig. 2. Gel filtration chromatogram of the *Pholiota squarrosa* Quel. AS 5.245 exopolysaccharide on a Sephadex G-100 column.

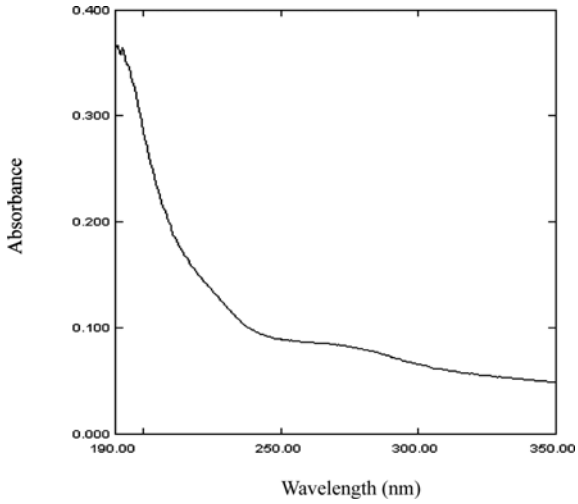

Fig. 3. The UV spectrum of PEPS-1.

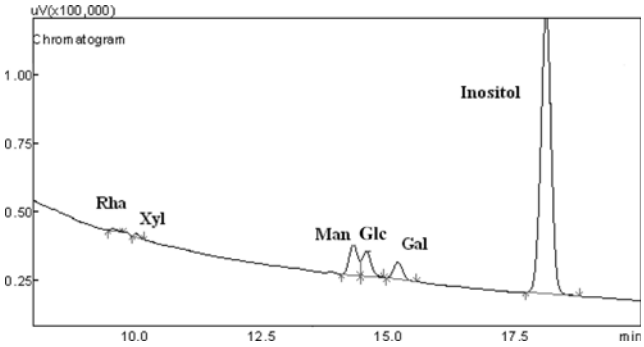

Fig. 4. Gas chromatogram of the monosaccharide composition PEPS-1.

number of different monomers present, polysaccharides can be divided into the 2 classes of homopolysaccharides, which contain only one kind of monosaccharide, and heteropolysaccharides, which contain 2 or more kinds of monosaccharide units (17). PEPS-1 was a heteropolysaccharide-mannoglucan based on the monosaccharide composition.

***In vivo* antitumor activity of PEPS-1** *In vivo* effects of PEPS-1 on tumor growth in Heps tumor-bearing mice were studied. Effects of the polysaccharide against Heps tumor cells in mice are summarized in Table 1. After KM mice were administered PEPS-1 at dosages of 50 mg/kg and 100 mg/kg, growth of implanted Heps tumors in mice was significantly (p<0.05) inhibited, compared with controls, with inhibition rates of 56.09 and 78.46%, respectively. The positive control group (5-Fu) had an inhibition rate of 67.48%. The inhibitory activity against Heps increased in a dose-dependent manner in PEPS-1 treated mice at both 50 and 100 mg/kg, in agreement with Pang *et al*. (18) that a polysaccharide from *Ganoderma lucidum* also dose-dependently inhibited growth of transplanted S180, Heps, and EAC tumors in mice.

Body weight gains of control and experimental mice during the experimental period are shown in Table 1. After 8 days of treatment, the ratios of the body weight gain of treated group mice were significantly (p<0.05) lower than that of the control mice, especially 5-Fu treated group mice. The ratio of body weight gain of PEPS-1 group mice at 50 mg/kg was 24.35%, which was significantly (p<0.05) higher than that of the 5-Fu treated group. Thus, PEPS-1 did not have the same toxicity as 5-Fu, which killed normal cells along with cancer cells (19).

The immune system plays an important role in antitumor defense (20). Polysaccharides exerted anti-tumor effects via activation of different immune responses in a host animal (21,22). The spleen and thymus indices reflect the immune functions of these important immune organs in an organism. The effects of PEPS-1 on spleen and thymus weights of Heps bearing mice are shown in Table 2. A significant (p<0.05) decrease in the spleen index was observed in 5-Fu group mice, compared with model control group mice, indicating that 5-Fu suppressed the immunological function in mice, as described in previous studies (23,24). 5-Fu has also been reported to exert an antitumor activity through toxicity that kills both normal and cancer cells (17). The mechanism of cytotoxicity of 5-FU has been ascribed to misincorporation of fluoronucleotides into RNA and DNA and to inhibition of the nucleotide synthetic enzyme thymidylate synthase (TS) (25). Unlike 5-Fu treated mice, PEPS-1 treatment group mice exhibited a stronger effect for the thymus index than mice of the negative control group. Especially for PEPS-1 treatment group mice at 100 mg/kg, a significant (p<0.05) increase in thymus and spleen indices was observed, compared with model control group mice.

Ooi and Liu (26) reported that mushroom polysaccharides exerted antitumor effects via activation of the immune responses of the host rather than by attacking cancer cells directly. These effects may be achieved through pathways, such as stimulation of natural killer cells, T-cells, B-cells, and macrophage-dependent immune system responses. Wasser (5) reported that some polysaccharides required an intact T-cell component to exert an antitumor activity and that the activity was mediated through a thymus-dependent immune mechanism. In this study, PEPS-1 exerted a stronger positive effect on the thymus index, and the *in vivo* antitumor effect was probably related to the thymus-dependent immune mechanism rather than to direct cytotoxicity against tumor cells.

It is well known that the Mw and monosaccharide

Table 1. Antitumor activities of PEPS-1against Heps solid tumor growth in KM mice

Groups	Tumor weight (g)	Tumor inhibition rate (%)	Ratio of body weight gain (%)
Model	2.46±0.82	–	33.33
PEPS-1 (50 mg/kg)	1.08±0.25*[1)]	56.09	24.35*
PEPS-1 (100 mg/kg)	0.53±0.15*	78.46	1.05*
5-Fu (25 mg/kg)	0.80±0.23*	67.48	14.50*

[1)]*Mean values within a column are significantly different against negative control by standard t-test at p<0.05.

Table 2. Effects of PEPS-1 on organ weights

Groups	Dose (mg/kg)	Spleen index	Thymus index
Model (negative)		104.41±23.05	18.91±1.11
PEPS-1	50	127.92±46.64	19.99±6.94
PEPS-1	100	172.01±89.66*[1)]	30.00±8.02*
5-Fu (positive)	10	77.39±21.51*	18.86±3.83

[1)]*Mean values within a column are significantly different against negative control by standard t-test at p<0.05.

composition are closely related to immunological and antitumor activities of natural polysaccharides (27). High Mw polysaccharides appear to be more effective than low Mw varieties (28). For example, polysaccharide-K (Krestin, PSK), a (25%) protein-bound polysaccharide extracted from mycelia of *C. versicolor* with a high Mw of 94×10^3 Da was approved in Japan for clinical use in human cancer treatment (29). PSK acts directly on tumor cells and indirectly in the host to boost cellular immunity (29). However, some low Mw polysaccharides, such as lentinan and schizophyllan, exhibited the same antitumor activities as high Mw varieties (17). Some reports have suggested that mushroom polysaccharides with high antitumor activities are mostly heteropolysaccharides (26), and for heteroglycans, differences in Mw had no obvious influence on activities (30). Results reported herein were in agreement with these previous reports. The Mw of the neutral heteropolysaccharide PEPS-1 was 3.26×10^4 Da with a good antitumor activity. PEPS-1 was composed primarily of the monosaccharids mannose, glucose, and galactose, which may be responsible for the high antitumor activity of PEPS-1 in Heps tumor-bearing mice.

Some study reported that some of the biological properties of *P. squarrosa* (Pers. *ex* Fr.) Quel. AS5.245 were due to the presence of low Mw compounds, such as proteins (31). The PEPS-1 polysaccharide from *P. squarrosa* (Pers. *ex* Fr.) Quel. AS5.245 exhibited a potent antitumor activity. The antitumor polysaccharide from *P. squarrosa* might be a heteropolysaccharide-mannoglucan consisting of Man:Glc:Gal (50:33:18 molar ratios) and PEPS-1 probably exerted an antitumor activity via stimulation of the immune response in a host. Detailed structure-function relationships and action mechanisms require further study.

Disclosure The authors declare no conflict of interest.

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