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Characterization of fungal sulfated polysaccharides and their synergistic anticancer effects with doxorubicin

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ABSTRACT

Sulfated polysaccharides (SPSs) from two edible fungal species, including two strains of *Antrodia cinnamomea* and *Poria cocos*, were isolated. Fucose, glucosamine, galactose, glucose, and mannose were the major sugars in the SPSs, and these SPSs had a high sulfate content. The area percentage of low-molecular-weight SPSs (1–100 kDa) covered almost half of the SPS mixture of the *A. cinnamomea* strains. In contrast, high-molecular-weight SPSs (>1000 kDa) of *P. cocos* covered a large proportion of the area at 30.06%. SPSs from *A. cinnamomea* B86 showed stronger inhibition of endothelial cell (EC) tube formation in an *in vitro* assay of angiogenesis, than did *A. cinnamomea* 35396 or *P. cocos*. The degree of sulfation paralleled their antiangiogenic activity. When tumor cells were concurrently exposed to doxorubicin (DOX) and fungal SPSs, SPSs synergistically increased the cytotoxicity of DOX to different degree up to 50-fold. Fungal SPSs may offer new applications for combinational-therapy drugs.

1. Introduction

For many years, interest has concentrated on sulfated polysaccharides (SPSs) as potentially useful, biologically active ingredients for pharmaceutical use. Drugs with a variety of biological activities, such as tumor suppression, anticoagulation, and antithrombosis, were developed (Miao et al., 2004; Roden et al., 1992; Veena, Josephine, Preetha, & Varalakshmi, 2007).

SPSs are defined as compounds with hemi-ester sulfate groups on a polysaccharide backbone. SPSs are considered an attractive class of compounds as drug candidates for anticancer therapies. There is now clear evidence that recognition of cell-surface heparan sulfate, with a structure similar to that of SPSs, is required for growth factor actions during angiogenic processes (Cohen et al., 1995; Vlodavsky, Miao, Medalion, Danagher, & Ron, 1996). Commercially available PI-88 is a mixture of highly sulfated, monophosphorylated mannose oligosaccharides, derived from the extracellular phosphomannan of the yeast *Pichia holstii* (Parish, Freeman, Brown, Francis, & Cowden, 1999), with potential antiangiogenic activity. Because SPSs can be easily commercially produced by submerged fermentation, it is worthwhile investigating this class of compounds for therapeutic purposes and to develop potential food supplements.

Physiologically active SPSs can be produced from commercially cultivated mushrooms. Due to the wide variety of biological effects elicited by bioactive SPSs, a current challenge is to investigate whether there are any differences and similarities in structural features of isolated SPSs which may account for certain biological effects of these compounds, including their ability to regulate inflammation, angiogenesis, apoptosis, and cell adhesion and to determine their anticarcinogenic efficacies. One of our previous studies was devoted to physiochemical characterization of SPSs from the fungus *Antrodia cinnamomea* (Polyporaceae) (Cheng, Huang, Lur, Kuo, & Lu, 2009). We also demonstrated that the SPSs from *A. cinnamomea* exhibit antiangiogenic and neuroprotective activities.

There is already considerable interest in the synergistic effects of SPS with antiviral and immunomodulatory agents. For example, SPSs from *Agaricus blazei* exhibited a synergistic antiviral effect with acyclovir for treating herpes infections and especially benefited patients who did not respond to acyclovir (de Sousa Cardozo et al., 2011). A higher sulfur content was correlated with a stronger inhibitory effect on the herpes simplex virus. In *Lucium barbarum*, a higher degree of sulfation of SPSs was reported to exhibit better immune activity, including generating antibodies earlier, accumulating them more quickly, and the effect lasting for a longer time. Also, its SPSs showed a synergistic effect with phytohemagglutinin in stimulating T lymphocyte proliferation (Wang et al., 2010). It is worthwhile investigating the synergistic effects of SPSs with anticancer agents.

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Angiogenesis is a multistep process that occurs early in tumor development and is rate limiting for tumor progression (Folkman, 2004). Angiogenesis and the development of metastases are intrinsically connected. The development of angiogenic inhibitors is an area that is ripe for expansion. In this study, the antiangiogenic activities of fungal SPSs were evaluated.

2. Materials and methods

2.1. Materials

Antrodia cinnamomea strains B86 and 35396 were a kind gift from Dr. Tun-Tschu Chang, Division of Forest Protection, Taiwan Forestry Research Institute, Taipei, Taiwan. *Poria cocos* (BCRC 36022) was purchased from the Bioresource Collection and Research Center (Hsinchu, Taiwan).

2.2. Liquid culture

Fungi were maintained on potato dextrose agar (PDA) slants and transferred to fresh medium at 3-week intervals. In each pasteurized Petri dish, 25 ml of PDA medium (39 g/L) was used and incubated at 28 °C for 19 days. Fine mycelia on the media surface were transferred to 800-ml culture flasks containing 100 ml of 24 g/L potato-dextrose-broth (PDB), with 20 g/L glucose (pH 5.6) at 28 °C. SPSs were isolated from 49-day-old cultures. Following incubation, mycelia were rapidly washed with 1 L of 250 mM NaCl during aspiration to remove contaminating exopolysaccharides. Samples were then lyophilized, and stored at 4 °C, and the dry weight of mycelia was measured.

2.3. Isolation of SPSs

SPSs were isolated according to Albano and Mourio (1986) with the following modifications. Lyophilized mycelia (1 g) were extracted with 40 ml of 0.1 M sodium acetate (pH 5.5), containing 5 mM cysteine, 100 mg papain, and 5 mM EDTA at 60 °C for 24 h. Supernatants were collected after being centrifuged at 2000 × *g* for 10 min at 4 °C, and another 100 mg papain in 40 ml of the same buffer containing 5 mM cysteine and 5 mM EDTA was added to the precipitate for another 24 h at 60 °C. The supernatants of the two extractions were collected, and a 3.75-fold volume of 95% ethanol was added, precipitated at 4 °C overnight, and spun at 9000 × *g* for 10 min at 4 °C, and the pellets were collected. Pellets were dried and resuspended in 20 ml of distilled water, dialyzed (MW 12–14 kDa) against distilled water overnight at 4 °C, and centrifuged at 9000 × *g* for 10 min; then the supernatant was collected and lyophilized before use.

2.4. Size-exclusion chromatography (SEC) of SPSs

An SPS solution in milli-Q water was diluted to give a concentration of 1 mg/ml and was then filtered through a 0.22-μm filter (Millipore, Billerica, MA, USA) before injection onto the SEC column. The flow rate was 0.5 ml/min, with deionized water as the eluent. A calibration curve was constructed using an authentic standard, Sodex P-82 series (Showa Denko America, Mentor, OH, USA) containing polymaltotriose with molecular weights of 78.8×10^4, 40.4×10^4, 21.2×10^4, 4.73×10^4, and 1.18×10^4 Da. The TriSec software program was used to acquire and analyze the Viscotek data. SEC signal detection was performed using a ViscoTek model TDA-3-1 relative viscometer (ViscoTek, Houston, Texas, USA).

2.5. Hydrolysis of SPSs

Acid hydrolysis of SPSs was carried out as follows. Lyophilized SPS (1 mg) was hydrolyzed with 4.95 N trifluoroacetic acid (TFA) at 80 °C in a heating block for 24 h. The mixture was cooled, evaporated, and then resuspended in milli-Q water.

2.6. High-performance anion-exchange chromatographic (HPAEC) analysis of SPSs

Hydrolysates of SPSs were separated by HPAEC (Dionex BioLC, Sunnyvale, CA, USA) equipped with a gradient pump, a pulsed amperometric detector (PAD-II) using a gold working electrode, and an anion-exchange column (Carbopac PA-10, 4.6 × 250 mm). Samples were applied with an autosampler (AS3500, SpectraSYSTEM) *via* a microinjection valve with a 200-μl sample loop. Monosaccharides were analyzed at an isocratic NaOH concentration of 18 mM at ambient temperature. Monosaccharides were identified and quantified by comparison to standards. Data were collected and integrated on a PeakNet system (Dionex). HPAEC standards of myo-inositol (99%), sorbitol (98%), fucose (99%), arabinose (99%), glucosamine (99%), galactose (99%), glucose (99.5%), mannose (99%), and fructose (99%) were purchased from Sigma (St. Louis, MO, USA).

2.7. Determination of sulfate ions (SO_4^{2-})

The method for estimating the liberated sulfate ions was based on Saito, Yamagata, & Suzuki (1968) with the following modifications. An aliquot (300 μl) of the acid hydrolysis of SPSs was pipetted into 700 μl of a $BaCl_2$-gelatin solution (see below). After mixing, the entire solution was allowed to stand for 10 min at room temperature. The absorbance of the test solution was measured against a blank at 360 nm. The $BaCl_2$-gelatin solution was prepared by dissolving 0.5 g gelatin in 100 ml of distilled water at 60 °C and allowing it to stand at 4 °C overnight. To this solution, 1.48 ml of 12.1 N HCl and 0.5 g $BaCl_2$ were added.

2.8. Cell culture

One endothelial cell line (human endothelial-like cells, Eahy926) and four cancer cell lines, MCF-7 (breast cancer), NCI-H460 (lung cancer), HT-29 (colon cancer), and CEM (leukemia), were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA) and were maintained in DMEM or RPMI medium (Life Technologies, Carlsbad, CA, USA) supplemented with 2 mM L-glutamine and 10% heat-inactivated fetal bovine serum (FBS) (Life Technologies) under standard culture conditions. The cell viability and cell number were determined by the Trypan blue dye-exclusion method.

2.9. Determination of cell viability

To assess cell viability, the alamar blue (AB) assay (dye purchased from Biosource International, Nivelles, Belgium) was used. Doxorubicin (DOX) was used as a positive control. Cells were treated with different SPSs with or without DOX for 48 h, and the viability was determined. This involved aspirating medium at the end of each treatment period and adding 100 μl of fresh medium containing 10% (v/v) AB to the control and treated wells. Plates were incubated at 37 °C for 6 h prior to measuring the absorbance at 540 and 595 nm wavelengths using a spectrophotometric plate reader (DYNEX Technologies, Chantilly, VA, USA). Experimental data were normalized to control values. Serial dilutions were performed to