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Characterization and biological functions of sulfated polysaccharides from sulfated-salt treatment of *Antrodia cinnamomea*

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ABSTRACT

Sulfated polysaccharides (SPSs) of *Antrodia cinnamomea* were extracted by an exhaustive papain digestion and characterized, and their biological functions were evaluated. In this study, we demonstrated the existence of SPSs in the medicinal fungus, *A. cinnamomea*. Since no SPSs had previously been identified in any fungal organism, we attempted to characterize those from *A. cinnamomea*. SPSs from *A. cinnamomea* inhibited *in vitro* Matrigel tube formation, in an angiogenesis model, in a dose-dependent manner. Furthermore, using serum deprivation-induced apoptosis in neuronal-like PC12 cells as a stress model, the SPSs of *A. cinnamomea* were effective in preventing serum-deprived apoptosis. Compositional analysis revealed that myo-inositol, fucose, galactose, and glucose were the neutral sugars in SPSs of *A. cinnamomea*, and these SPSs had a high sulfate content. The sulfation degree paralleled their antiangiogenic and neuroprotective activities. In this work, we report novel data on the structure, antiangiogenic, and neuroprotective effects of these fungal SPSs.

1. Introduction

Sulfated polysaccharides (SPSs) are found in the structural skeleton of invertebrate connective tissues [1–5]. The physiological function of SPSs is to support the skeletal structure. SPSs are considered to be an attractive class of compounds as drug candidates for anticancer therapies. There is now clear evidence that recognition of cell-surface heparan sulfate, a similar structure of SPS, is required for growth factor actions during angiogenic process [6,7]. Commercially available PI-88 is a mixture of highly sulfated, monophosphorylated mannose oligosaccharides, derived from the extracellular phosphomannan of the yeast *Pichia holstii* [8], with potential antiangiogenic activity. PI-88 is currently in phase III clinical trials in the US. It is worthwhile screening this class of compounds found in nature.

Antrodia cinnamomea, also known by its Chinese name, *niu-chang-chin*, in Taiwan, is a medicinal fungus of the family Polyporaceae that grows slowly in the inner cavity of the camphor tree, *Cinnamomum kanehirai* [9]. It is an indigenous and rare species in Taiwan. It has recently been used in the formulation of neutraceuticals and functional foods due to many of its bioactive constituents being discovered, and are commercially available in Taiwan. *A. cinnamomea* has not only been utilized to treat a wide variety of diseases, but has also drawn the attention of the pharmaceutical industry. Traditionally, it has been used to treat intoxication caused by food, alcohol, and drugs as well as to treat diarrhea, abdominal pain, hypertension, itchy skin, and tumorigenic diseases [10]. Chemical compounds found in *A. cinnamomea* include sesquiterpene lactone, steroids, and triterpenoids [11–14]. Recently, differential extracts of *A. cinnamomea* have been shown to exert antioxidative [15–17], vasorelaxation [18], anti-inflammatory [19], and antihepatic effects [20]. Attemptes to obtain optimal submerged culture conditions for polysaccharide production in this species was reported [21].

Angiogenesis is a dynamic process of endothelial proliferation and differentiation. Cancer cells are able to produce large amounts of several angiogenic factors including basic-fibroblast-like growth factor (bFGF) and vascular endothelial growth factor (VEGF), interleukin 8 (IL-8), transforming growth factors-ß (TGF-ß), and others that cause endothelial cell recruitment and proliferation [22]. Tumors with high angiogenic activity have been connected with aggressiveness and poor patient survival [23–25], and blocking the nutrient supply to cause a rapid and selective shutdown of new blood vessel and tumor growth is an effective cancer therapy. Fungal polysaccharopeptides (PSPs) from *Coriolus versicolor* are commercially available and have inhibitory effects on tumor angiogenesis and tumor growth in mice [26]. For the ease of commercial production of polysaccharides in submerged fermentation, it is worthwhile to investigate this class of compounds to develop a potential food supplement and for therapeutic uses.

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Recently, we showed that *A. cinnamomea* is effective in preventing serum-deprived PC12 cell apoptosis [27–29], suggesting a neuroprotective role. Neuronal death induced by apoptosis is a normal aspect of development in which it seems that the death program is triggered by the failure of a given neuron to receive limited supplies of target-derived neurotrophic factors. In the post-developmental period, neurons also undergo apoptotic death when deprived of appropriate trophic factors or are subjected to a variety of stresses and injuries. The rat PC12 cell line is a commonly used model for studies of neuronal differentiation and cell death. Apoptosis may occur when triggered by deprivation of either serum [30] or trophic factor/nerve growth factor (NGF) [31,32]. In this study, serum deprivation-induced PC12 cell death was used as an apoptotic model to investigate the mechanism of the extract of *A. cinnamomea*.

In a previous study, strain CCRC 35396 of *A. cinnamomea* grew fastest among all of the strains in our laboratory, and is rich in adenosine. It was also proven that adenosine can prevent cells from undergoing serum deprivation-induced apoptosis [28]. Therefore, in the present study, the antiangiogenic and antiapoptotic activity of SPSs from *A. cinnamomea* 35396 was evaluated as a reference for potential applications as health foods or drugs.

2. Materials and methods

2.1. Reagents and cell culture

All reagents were purchased from Sigma Chemical (St. Louis, MO, USA) except where otherwise specified. Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), and horse serum were purchased from HyClone (Logan, UT, USA). PC12 cells were maintained in DMEM supplemented with 10% (v/v) horse serum and 5% (v/v) FBS and incubated in a CO_2 incubator (5%) at 37 °C.

2.2. Liquid culture of A. cinnamomea

An *A. cinnamomea* isolate, strain 35396, was a generous gift from fungal specialist Dr. T.T. Chang (Division of Forest Protection, Taiwan Forest Research Institute, Taipei, Taiwan). 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. A fungus was inoculated at the center of a Petri dish which was then incubated at 28 °C for 19 days. For liquid culture, 19-day-old seeding mycelium of *A. cinnamomea* was transferred to 100 ml of 24 g/l potato dextrose broth (PDB), and 20 g/l glucose (pH 5.6) with $MgSO_4$ or $MnSO_4$ in test concentrations of 0.05–0.50% for 49 days. At the end of the incubation, mycelia were rapidly washed with 1 l of 250 mM NaCl using an aspirator-suction system to remove the contaminating culture medium. Samples were then lyophilized before use in the further experiments.

2.3. Preparation of SPSs from A. cinnamomea

SPSs were isolated according to Albano and Mourao [2] 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 centrifugation 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. The pellets were dried and resuspended in 20 ml of distilled water, dialyzed (MW 12,000–14,000 Da) 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. Preparation of polysaccharides (PS) from A. cinnamomea

Polysaccharides (denoted non-sulfated PS) extracted from the same species under same culture medium without the addition of $MgSO_4$ or $MnSO_4$ were used as negative control. The non-sulfated PS were isolated from lyophilized mycelia by hot water at 80 °C in a 1:100 (w/w) ratio for 6 h twice and cooled, and then four volumes of 95% ethanol were added and precipitated overnight at 4 °C. The precipitated polysaccharides were collected by spinning at 9000 × *g* for 20 min and lyophilized, resulting in a crude polysaccharide sample.

2.5. Cell viability assay

Survival was assessed by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay as described by Mosmann [33]. Cells growing on 150-mm plates were washed three times with PBS and resuspended in DMEM. Suspended cells were plated on 96-well plates and treated with the indicated reagent(s) (1×10^4 cells/200 μl/well). After incubation for 24 h, 20 μl of MTT stock (5 mg/ml) was added to the medium and incubated at 37 °C for 3 h. After discarding the medium, DMSO (100 μl) was applied to the well to dissolve the formazan crystals derived from the mitochondrial cleavage of the tetrazolium ring by live cells. The absorbance at 570/630 nm, which is highly correlated with the number of cells in each well was measured on a micro-ELISA reader. Cell viability was expressed as a percentage of the results of the MTT metabolism assay (o.d. 570/630 nm) measured in the serum-containing group.

2.6. Matrigel endothelial cell (EC) tube formation assays

An EC tube formation assay was modified from a previously described method [34]. Matrigel (12.5 mg/ml) was thawed at 4 °C, and 50 μl was quickly added to each well of a 96-well plate and allowed to solidify for 10 min at 37 °C. Once the gel had solidified, the wells were incubated for 30 min with ECs (30,000 cells/well). After adhesion of the cells, the medium was removed and replaced by fresh medium supplemented with VEGF with or without SPSs and incubated at 37 °C for 24 h. The growing tubes were visualized with an inverted Zeiss microscope (Germany) at a magnification of 10×.

2.7. Size-exclusion chromatography (SEC) of polysaccharides

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, Bedford, MA) before injection into 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) 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 signals were detected using a ViscoTek model TDA-3-1 relative viscometer (Viscotek, Houston, TX).

2.8. Hydrolysis of SPSs

Acid hydrolysis of the SPSs was carried out as follows. One milligram of lyophilized SPSs was hydrolyzed with 4.95 N trifluoroacetic acid (TFA) at 80 °C in a heating block for 4 h. The mixture was cooled and evaporated to remove the acid and then resuspended in milli-Q water.

2.9. High-performance anion-exchange chromatographic (HPAEC) analysis of the carbohydrate composition of the SPSs

After acid hydrolysis, the SPSs were separated on an HPAEC system (Dionex BioLC, Sunnyvale, CA) 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 mm × 250 mm, Dionex, Sunnyvale, CA). Samples were applied using an autosampler (AS3500, SpectraSYSTEM, Thermo Separation Products Inc., Riviera Beach, FL) via a microinjection valve with a 200-μl sample loop. The monosaccharides were analyzed at an isocratic NaOH concentration of 18 mM at ambient temperature. Identification and quantification of the monosaccharides were made in comparison with standards. Data were collected and integrated on a PeakNet system (Dionex, Sunnyvale, CA).

2.10. Determination of sulfate ion (SO_4^{2-})

The method for estimating the liberated sulfate ions was based on Saito et al. [35] with the following modifications. An aliquot (300 μl) of the acid hydrolysis of SPSs was pipetted into 700 μl of the $BaCl_2$-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. A $BaCl_2$-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 12.1N HCl and 0.5 g $BaCl_2$ were added.

2.11. Statistical analysis

Statistical analysis was performed using Student's *t*-test. Data are presented as the mean ± S.E.M. Statistical significance was defined as $p < 0.05$.

3. Results

3.1. Time-course study of growth

To maximize production of the fungus, a time-course study was performed on the dry mass accumulation. In the culture period between 7 and 14 days, a linear phase of growth was observed. Beyond 21 days, the culture entered a senescent phase (Fig. 1).

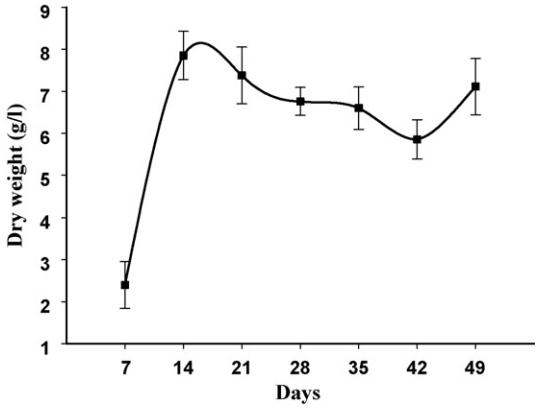

Fig. 1. Time course of the growth of *A. cinnamomea*. Data are presented as the mean ± S.E. from three independent experiments.

3.2. Influence of $MgSO_4$ and $MnSO_4$ on mycelial growth and SPS production

To evaluate the effects of $MgSO_4$ or $MnSO_4$ on mycelial growth and SPS production, the dry biomass and yield of SPSs were measured (Fig. 2). Comparisons were made between control and $MgSO_4$- and $MnSO_4$-treated cultures in the dose range of 0.05–0.5% of 49-day-cultured mycelia of *A. cinnamomea*. All $MgSO_4$ treatments showed significantly enhanced mycelial growth compared to the control, whereas $MnSO_4$ treatment inhibited mycelial growth. All $MgSO_4$ treatments showed higher levels of dry mass accumulation than those with $MnSO_4$ treatment (Fig. 2A). $MgSO_4$- and $MnSO_4$-treated mycelia exhibited no differences in SPS production (Fig. 2B).

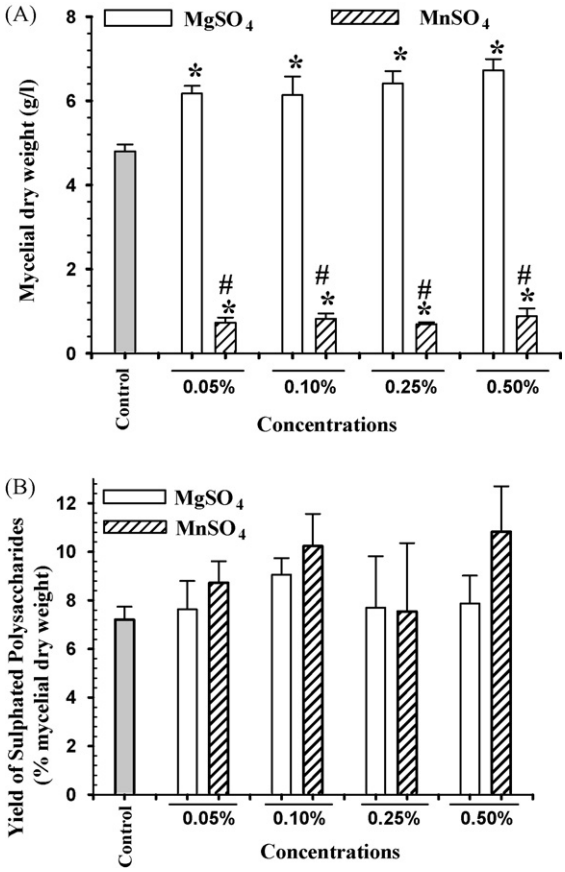

Fig. 2. Effect of $MgSO_4$ and $MnSO_4$ on the (A) dry weight; (B) yield of sulfated polysaccharides (SPSs) for 49 day-cultured *A. cinnamomea*. Data are presented as the mean ± S.D. ($n = 5$). $^*p < 0.05$, compared with control by *t*-test. $^*p < 0.05$ vs. the control, $^\#p < 0.05$ vs. $MgSO_4$.

3.3. Effects of SPSs from A. cinnamomea on EC tube formation

To study the effects of SPSs from *A. cinnamomea* on angiogenesis, an in vitro assay was performed to evaluate EC tube formation in Matrigel. Serial dilutions of SPSs from *A. cinnamomea* were evaluated for their effects on VEGF-induced angiogenesis revealed by tube formation on Matrigel (Fig. 3). SPSs from $MnSO_4$-treated *A. cinnamomea* showed stronger inhibition of tube formation than did $MgSO_4$-treated *A. cinnamomea*. SPSs of both $MgSO_4$- and $MnSO_4$-treated *A. cinnamomea* showed dose-dependent inhibition of tube

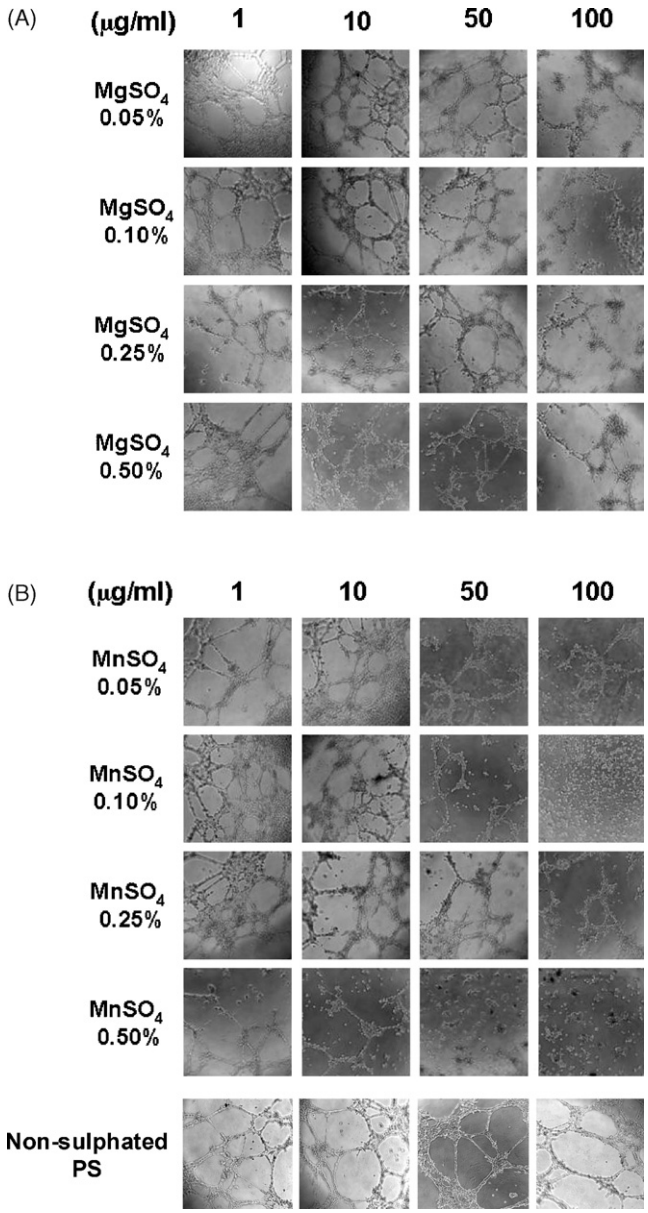

Fig. 3. Influences of sulfated polysaccharides (SPSs) of *A. cinnamomea* on endothelial cell (EC) tube formation. ECs were seeded onto Matrigel and cultured for 24 h with vascular endothelial growth factor (VEGF) with or without pretreatment with serial concentrations of SPSs from *A. cinnamomea*. Capillary tube formation on Matrigel was visualized with an inverted Zeiss microscope at a magnification of 10×. Non-sulfated PS was used as a negative control.

formation. The effect of non-sulfated polysaccharide (PS) on matrigel formation was almost not affected up to 100 μM concentration. This assay revealed that EC tube formation was attenuated by treatment with SPSs from *A. cinnamomea* for 24 h (Fig. 3).

3.4. Effects of SPSs from A. cinnamomea on serum deprivation-induced PC12 cell apoptosis

SPSs from different concentrations of $MnSO_4$-treated *A. cinnamomea* were used to treat serum-deprived PC12 cells, and we found that only 0.5% $MnSO_4$ of SPSs dose-dependently prevented PC12 cells from serum deprivation-induced apoptosis (Fig. 4B). However, SPSs from $MgSO_4$ treatment showed no protective effects (Fig. 4A).

3.5. Effects of $MgSO_4$ and $MnSO_4$ on the molecular weight distribution and chemical composition of SPSs from A. cinnamomea

To elucidate the effects of $MgSO_4$ and $MnSO_4$ on the structure of SPSs, SPSs were characterized according to their molecular weight distributions. The left panel of Fig. 5 shows results of $MgSO_4$ treatment, and the right panel shows results of $MnSO_4$ treatment. The molecular weight distributions of the SPS preparations were characterized as very high molecular weight (>5000 kDa, peak A), high molecular weight (>1000 kDa, peak B), medium high molecular weight (>100 kDa, peaks C–E), and low molecular weight (<100 kDa, peaks F and G). The results showed that the synthesis of very-high-molecular weight SPSs of peak A increased when treated with $MgSO_4$ at concentrations exceeding 0.1%, and that of high-molecular-weight SPSs of peak B decreased. With $MgSO_4$ treatment at concentrations of 0.05%, 0.1%, and 0.25%, the sums of the percent area of the medium-high-molecular-weight SPSs of peaks C, D, and E had values of 31.98%, 29.49%, and 30.06%, respectively. Almost no difference in all the tested concentrations of $MgSO_4$ was shown for this molecular size range. Molecules of <100 kDa (peaks F and G) in size accounted for half of the SPS population. With $MnSO_4$ treatment at concentrations of 0.1%, 0.25%, and 0.5%, the sums of the percent area showed that the medium-high-molecular-weight SPS peaks C, D, and E decreased to values of 36.77%, 34.03%, and 27.96%, respectively. With 0.5% $MnSO_4$ treatment, a new species of SPS was synthesized with an Mn value of 90.74 kDa (peak F); this SPS population was not present with the other $MnSO_4$ treatments, and the low-molecular-weight peak G with an Mn value of 6.69 kDa represented the major population of SPSs. In contrast, the molecular weight distribution of non-sulfated PS was different from all the SPSs in that the molecular weight in the rang of 50–300 kDa was absent.

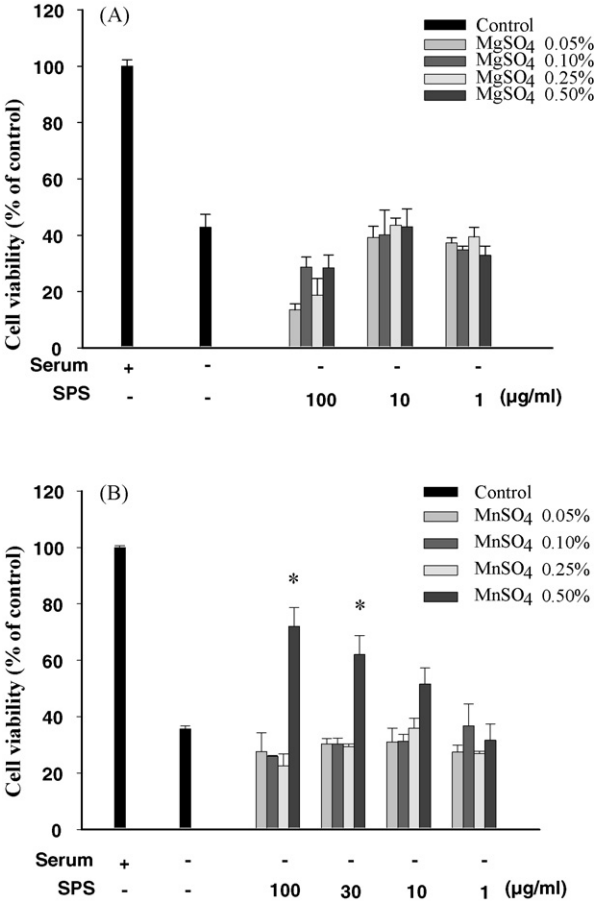

Fig. 4. Effect of $MgSO_4$ and $MnSO_4$ treatment on sulfated polysaccharides (SPSs) from *A. cinnamomea* on serum deprivation-induced PC12 cell apoptosis. Serum-deprived PC12 cells were treated with or without different concentrations of (A) $MgSO_4$-treated and (B) $MnSO_4$-treated SPSs for 24 h. Cell viability was expressed as a percentage of the results of the MTT assay measured in the serum-contained control group. Data points represent the mean ± S.E.M. of at least three independent experiments (n = 3–6). $^*p < 0.05$ compared to the serum-deprived group.

The chemical compositions of the SPSs were determined after each SPS was completely hydrolyzed (Table 1). The results showed that myo-inositol, galactose, and glucose were the predominant sugars in SPSs of *A. camphorata*. The sulfate content increased with an increasing concentration of both $MgSO_4$ and $MnSO_4$. In contrast, the sugar composition of non-sulfated PS was different from all the SPSs in that the concentration of fucose, galctose and glucose were higher than all the SPSs and sulfate was absent. To elucidate the most potent components in terms of antiangiogenic and neuroprotective activities, comparisons were made between the effective compounds treated with $MnSO_4$, and the non-effective ones treated with $MgSO_4$ in all the corresponding dosages. Sulfate may play important roles for the activity.

4. Discussion

To our knowledge, this is the first attempt to purify SPSs from a fungal species, *A. cinnamomea*. In this report, we used *the* CCRC35396 strain of *A. cinnamomea* and discuss dissimilarities in chemical and biological features between fungal and invertebrate SPSs. For our study to be successful, the physiological nature and chemical architecture of the bioactive domains of the SPSs had to be determined. Herein, a sulfated salt (SO_4^{2-}) was used to stress the mycelial culture to derive our data.

In this study, there were significant qualitative changes in the sugar compositions, and degree of sulfation (Table 1) between $MgSO_4$ and $MnSO_4$ treatments of *A. cinnamomea*. It might have resulted in different biological functions. Our results indicated the same carbohydrate pattern as in the sea cucumber. Fucose was the major sugar species in SPSs of the sea cucumber [36]. Our results indicated that SPSs from *A. cinnamomea* contained common sugars (fucose, galactosamine, glucose, and galactose) which are also found in many invertebrate SPSs [2,37]. Nevertheless, the SPSs from *A. cinnamomea* differed from those of other species due to their relatively less-complex composition, as they mainly contained galactose and glucose (Table 1). Our findings indicated that SPSs from 0.5% $MnSO_4$-treated mycelia showed the most potent antiangiogenic (Fig. 3) and antiapoptotic activities (Fig. 4). However, non-sulfated PS did not exhibited angiogenic activity under the same concentration. The degree of sulfation of the SPSs increased as the dose of $MnSO_4$ increased. The sulfate content of SPSs from 0.5% $MnSO_4$-treated mycelia was the highest among all treatments. This suggests that the degree of sulfation affected the antiangiogenic and neuroprotective properties. These results are

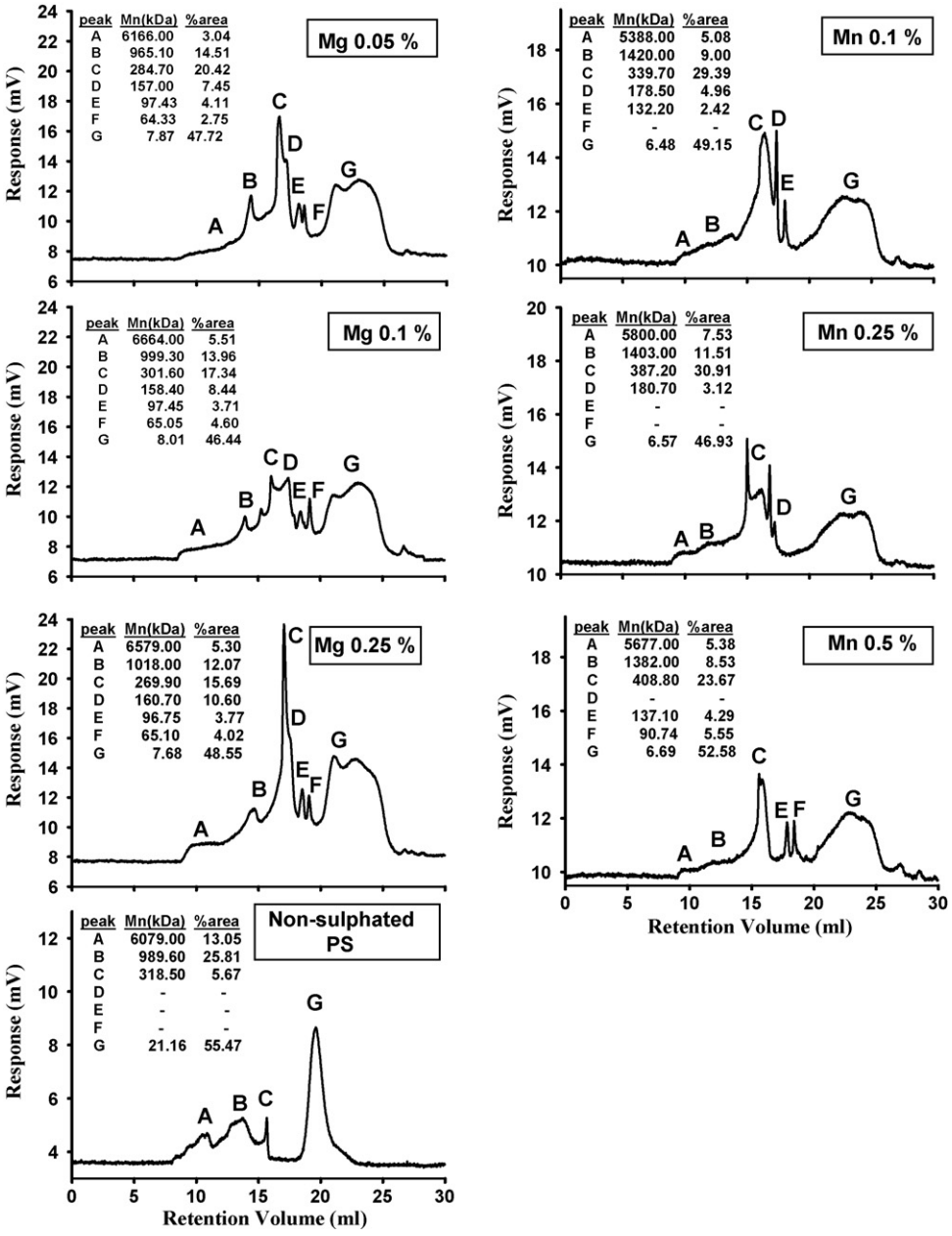

Fig. 5. Effect of $MgSO_4$ and $MnSO_4$ treatment on the molecular weight distribution of sulfated polysaccharides (SPSs) isolated from *A. cinnamomea*. SPSs were isolated from 49-day-old $MgSO_4$- and $MnSO_4$-treated mycelia of *A. cinnamomea*. Size-exclusion chromatography (SEC) was performed using a ViscoTek model TDA-3-1 relative viscometer (Viscotek). A polysaccharide solution in milli-Q water was diluted to give a final concentration of 1 mg/ml for the determination. Non-sulfated PS was used as a negative control.

consistent with those of Parish et al. [8] who found that the inhibitory activity against human angiogenesis of SPSs was critically dependent on the chain length and degree of sulfation. Highly sulfated linear oligosaccharides of five or more monosaccharides in length showed the greatest activity.

Characterization of the molecular weight distribution of these SPSs showed that a new species was synthesized with an Mn value of 90.74 kDa, and the low-molecular-weight peak G with an Mn value of 6.69 kDa represented the major population of SPSs from 0.5% $MnSO_4$-treated mycelia (Fig. 5). These findings are in accord with the results of a previous study of synthetic sulfated oligosaccharides, which block heparan sulfate recognition by growth factors and inhibit cleavage of heparan sulfate by heparanase. Low-molecular-weight mimics of heparan sulfate showed the greatest effectiveness because it is now believed that cell surface heparan sulfates aid dimerization of growth factor receptors by growth factors [7].

Polysaccharides are getting increasing attention for therapeutic use in recent years. After extensive clinical trials, polysaccharopeptide Krestin (PSK) was approved for use in Japan in 1977, and polysaccharopeptide (PSP) appeared on the market about 10 years later [38]. In addition to fungal polysaccharides, low-molecular-weight sulfated *Escherichia coli* K5 polysaccharide exerts antiangiogenic activity as a fibroblast growth factor (FGF) antagonist [39]. The K5 polysaccharide has the same structure as the heparin precursor. Heparin sulfate proteoglycans (HSPGs) modulate the binding of VEGF and FGFs with their respective receptors. HSPGs also stabilize the FGF-2/FGF receptor complex and VEGF/VEGF receptor complex. Regardless of the polysaccharide structure or sulfation degree, both contribute to the antiangiogenic activity. Angiogenic inhibitors may

Table 1
Compositional analysis of the SPS from cultured mycelia of *A. cinnamomea*.

Treatment	SPS (μmol/g)					
	Inositol	Fucose	Galactosamine	Galactose	Glucose	Sulfate[a]
Control	27.70 ± 5.86	18.08 ± 0.40	1.00 ± 0.48	40.02 ± 2.35	26.21 ± 1.54	301.78 ± 0.89
$MgSO_4$ 0.05%	15.88 ± 3.69	15.81 ± 0.49	15.72 ± 2.92	29.10 ± 0.90	61.22 ± 5.31	382.89 ± 2.44
$MgSO_4$ 0.10%	17.05 ± 6.33	9.49 ± 0.35	–	46.46 ± 8.34	47.19 ± 5.32	382.22 ± 0.89
$MgSO_4$ 0.25%	15.48 ± 9.43	23.93 ± 13.48	–	13.24 ± 3.14	24.29 ± 1.31	401.11 ± 1.74
$MgSO_4$ 0.50%	8.09 ± 0.03	3.91 ± 0.03	1.03 ± 0.03	7.00 ± 0.03	28.44 ± 0.10	426.50 ± 0.67
$MnSO_4$ 0.05%	16.61 ± 5.97	6.00 ± 4.13	4.23 ± 1.05	106.36 ± 7.31	43.41 ± 2.89	701.11 ± 2.07
$MnSO_4$ 0.10%	12.65 ± 0.33	2.82 ± 0.10	7.44 ± 0.62	127.51 ± 4.33	21.08 ± 0.87	964.00 ± 1.39
$MnSO_4$ 0.25%	15.67 ± 3.93	5.48 ± 1.41	7.64 ± 0.88	112.45 ± 8.06	39.14 ± 2.48	1044.66 ± 3.63
$MnSO_4$ 0.50%	10.11 ± 4.94	3.10 ± 0.39	10.12 ± 0.92	114.53 ± 0.05	25.12 ± 0.73	1075.11 ± 5.39
Non-sulfated PS	18.47 ± 0.93	115.78 ± 1.43	–	408.71 ± 7.67	101.57 ± 2.57	–

[a] Sulfate was determined by the $BaCl_2$-gelatin method [35].

possibly interfere with FGF-2 or VEGF activity in two ways: either directly by blocking the interaction of VEGF or FGF with their receptors or by disrupting the bystander effect of HSPGs for efficient growth factor binding. Therefore, in this study, the possible mechanism of angiogenesis inhibition or neuroprotective activity of SPSs from *A. cinnamomea* might have been due to the similar structural characteristics to sulfated proteoglycans.

In conclusion, this investigation of the existence of antiangiogenic and neuroprotective effects of fungal SPSs is truly novel and presents important findings. We identified that SPSs simultaneously inhibited angiogenesis by inhibition of EC tube formation and neuroprotective activities. However, the physiological significance of fungal SPSs is not completely understood. Do compositional differences in the sugars, sulfation degree, or polysaccharide structure cause the different biological functions between invertebrate and fungal SPSs? This study describes the successful use of an in vitro approach to study fungal SPSs as drug candidates. Further study should be performed to validate the specific active components.

Acknowledgements

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