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Fermentation optimization, characterization and bioactivity of exopolysaccharides from *Funalia trogii*

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ABSTRACT

Optimization of culture conditions for exopolysaccharide (EPS) by *Funalia trogii* in submerged culture was investigated using one-factor-at-a-time method and uniform design (UD). Under the optimized conditions, the maximum concentration of EPS was 8.68 g/l. After EPS was deproteinized by Sevag method, two groups of EPSs (designated as Fr-I and Fr-II) were obtained from the culture filtrates by gel filtration chromatography on Sepharose CL-6B. Furthermore, EPSs were characterized by size exclusion chromatography (SEC) coupled with a multiangle laser-light scattering (MALLS) and refractive index (RI) detector system. The weight-average molar masses of the Fr-I and Fr-II were determined to be 1.007×10^5 and 2.393×10^4 g/mol, respectively. The root mean square (RMS) radii for both peaks ranged from 9.7 to 10.8 nm with no clear trends. Pharmacology experiments indicated *F. trogii* EPS were useful to the therapy of free radical injury and cancer diseases.

1. Introduction

During the past several decades, much interest has been generated in extracellular polysaccharides (EPS) produced by submerged cultures of numerous mushrooms because they have biological and pharmacological activities including anti-tumor, antioxidant, hypoglycemic activities, etc. (Han et al., 2006; Li, Chen, Wang, Tian, & Zhang, 2010; Song, Lin, Yang, & Hu, 2008; Zhao, Xie, et al., 2012). Submerged culture of medical mushrooms obviously gives rise to potential advantages of effective EPS production in a compact space and shorter time without significant contamination risk (Kachlishvili, Penninckx, Tsiklauri, & Elisashvili, 2005; Sam & Yesilada, 2001).

Funalia trogii also called *Trametes trogii* is a white-rot basidiomycete that grows on lignin-bearing woods. It is capable of softening woody chips and straw during growth by degrading lignin using degradative enzymes including peroxidases and polyphenol oxidases (laccases) (Asma & Yesilada, 2002; Kachlishvili et al., 2005; Sam & Yesilada, 2001). *F. trogii* has been well known due to its various industrial applications including degradation of a variety of structurally diverse pollutants (Kahraman & Yesilada, 2003), decolorization of recalcitrant effluents such as olive oil mill and alcohol factory wastewater (Yesilada, Sik, & Sam, 1998) and removal of heavy metals like mercury (II) cations from industrial waste water (Arica, Bayramoglu, Yılmaz, Bekta, & Genç, 2004). Recently years, it has been found that *F. trogii* extract posses notable biological activities, such as antitumor and cytotoxic activities (Mazmanc et al., 2011; Rashid et al., 2011).

Some investigators have attempted to obtain optimal submerged or solid-state culture conditions for targeted degradative enzymes production by *F. trogii* (Kachlishvili et al., 2005; Sam & Yesilada, 2001). However, to the best of our knowledge, the nutritional requirements and environmental conditions for EPS production by submerged culture of *F. trogii* have not been demonstrated.

Medium optimization by the one-factor-at-a-time method involves changing one independent variable (i.e., nutrients, temperature, pH, etc.) while fixing the others at certain levels. This method is not only time-consuming but also often leads to an incomplete understanding of the behavior of the system, resulting in confusion and a lack of predictive ability. Hence, as a more practical method, the uniform design was employed to study relationships between medium components and their effect on EPS production.

It has been reported that the molecular weight, chemical composition, branching degree and conformation of the polysaccharides significantly affect their structure, macromolecular assembly, biological activities (Han et al., 2006; Li et al., 2010; Rashid et al., 2011; Song et al., 2008). In this regard, data of detailed molecular characterization are essentially required for elucidating the relationship between physiochemical properties and physiological functions.

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Therefore, the purpose of this study is first to optimize the submerged culture conditions to produce EPS by *F. trogii* by the one-factor-at-a-time method and uniform design. Subsequently, the pure EPSs were isolated by gel filtration chromatography, and the molecular features were investigated by a SEC/MALLS system. Then, in vitro antioxidant and antitumor activities of *F. trogii* EPS were investigated.

2. Materials and methods

2.1. Microorganism and media

Microorganism of *F. trogii* was collected by our own laboratory and was used throughout the study. The stock culture was maintained on potato dextrose agar (PDA) slants. Slants were incubated at 25 °C for 6 d and stored at 4 °C. The seed culture was grown in a 250 ml flask containing 50 ml of basal medium (0.3% peptone and 3% glucose) at 26 °C on a rotary shaker incubator (150 rev min^{-1}) for 4 d. The flask culture experiments were performed in a 250 ml flask containing 50 ml of medium inoculated with 4% (v/v) of the seed culture.

2.2. Inoculum preparation

F. trogii was initially grown on PDA medium in a petri dish, and then transferred into the seed medium by punching out 5 mm of the agar plate culture with a cutter (Kim, Hwang, et al., 2003; Kim, Xu, et al., 2003).

2.3. Analytical methods

Samples collected at various intervals from shake flasks were vacuum filtered by vacuum filtration. The resulting culture filtrate was mixed with 4 volumes of absolute ethanol, stirred vigorously and kept overnight at 4 °C. The precipitated EPS was centrifuged at speed of 10,000 rpm for 15 min using a refrigerated centrifuge separator and the supernatant was discarded. The precipitate of EPS was estimated through phenol–sulfuric acid method. The dry weight of mycelium was measured after repeated washing of the mycelial pellet with distilled water and drying at 70 °C overnight.

2.4. Purification of EPS

The ethanol precipitates of the crude EPS treated with Sevag reagent (1:4 n-butanol/chloroform, v/v) to remove most of proteins. After removing the proteins and Sevag reagent by centrifugation, the water phase was dialyzed against distilled water and lyophilized to yield EPS. The EPS were dissolved in 0.2 M NaCl buffer, and loaded onto a Sepharose CL-6B column (2.4 cm × 100 cm, Sigma Chemical Co., St. Louis, MO). The column was eluted with the same buffer at a flow rate of 0.6 ml/min. Protein concentration was determined according to the Bradford method (Bradford, 1976) using bovine serum albumin as the standard. The total sugar content in the EPS was determined by phenol sulfuric acid method using glucose as the standard (DuBois, Gilles, Hamilton, Rebers, & Smith, 1956). The protein moiety in the EPS was monitored by measuring the absorbance at 280 nm, whilst the carbohydrate moiety was monitored at 480 nm.

2.5. Analysis of amino acids

Total protein was determined by the Lowry method with bovine serum albumin as the standard (Lowry, Rosebrough, Farr, & Randall, 1951). The composition of amino acids was analyzed by amino acid analysis (Sykam S433; Sykam, Eresing, Germany).

2.6. SEC/MALLS analysis

The molecular weights of the EPS were estimated by SEC coupled with multi-angle static laser light scattering detection (MALLS; DWAN EOS equipped with a GaAs laser at 690 nm (λ), Wyatt Technology, Santa Babara, CA) and a Refractive Index (RI) detector (Optilab rEX, Wyatt Technology, Santa Barbara, CA). The EPS samples were dissolved in a 0.1 M PBS buffer (pH 6.8) containing 0.04% diaminotetraacetic acid–disodium salt (Na_2EDTA) and 0.01% sodium azide and filtered through 0.025 μm filter membranes (Millex HV type, Millipore Co., Bedford, MA) prior to injection into the SEC/MALLS system. The chromatographic system consisted of a degasser (Degasys, DG-1200, uniflow, HPLC Technology, Macclesfield, UK), a SSI 222D pump (Scientific Systems, State College, PA, USA) single-piston isocratic, pulse-dampened HPLC pump (Model 590 Programmable Solvent Delivery Module, Waters Co., Milford, MA), an injection valve (Rheodyne, Inc., Cotati, CA) fitted with a 500 μl loop, and two SEC columns (Shodex OH Pack SB-803 and 805 HQ, JM Science Inc., Buffalo, NY) connected in series. The flow rate was 0.75 ml/min and the injection volume and concentration was 100 ml and 2 mg/ml, respectively. During the calculation of molecular weights of each EPS, the value of dn/dc (specific refractive index increment) was used from the data in literature (Jumel, Fiebrig, & Stephen, 1996), in which the estimated dn/dc was 0.14 ml/g. Calculations of molecular weight and root mean square (RMS) radius of gyration for each EPS were performed by the Astra 4.72 software (Wyatt Technology). The RMS radii of each polysaccharide were determined from the slope by extrapolation of the first-order Debye plot (Astafieva, Eberlein, & Wang, 1996). The gross conformation of EPS in aqueous solution could be identified from the double logarithmic plot of RMS radius vs. molecular mass of EPS according to the following equations:

$$\text{Spheres}: \quad r_i^3 \propto M_i \rightarrow \log r_i = k + \frac{1}{3}\log M_i$$

$$\text{Random coils}: \quad r_i^3 \propto M_i \rightarrow \log r_i = k + \frac{1}{2}\log M_i$$

$$\text{Rigid rods}: \quad r_i^3 \propto M_i \rightarrow \log r_i = k + \log M_i$$

where r_i is the RMS radius of an EPS molecule, M_i is the molar mass of EPS, k is the intercept at the Y-axis (RMS radius), and 1/3, 1/2, and 1 are the critical slope values for determining the molecular conformation of each EPS (Hwang, Kim, Xu, Choi, & Yun, 2003; Kim, Xu, et al., 2003; Lim et al., 2005).

2.7. Antioxidant activity

2.7.1. DPPH radical scavenging activity

The antioxidant activity of the polysaccharides, based on the scavenging activity of the stable 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical, was determined by the method described by Eloff, Katerere, and McGaw (2008). The different amount of EPS was dissolved in distilled water to make different concentration solution. Two milliliters of 0.1 g/l DPPH in 50% ethanol was added to 2 ml of the EPS solution. The absorbance was measured at 517 nm after 20 min of incubation at 25 °C. 50% ethanol instead of DPPH was used for the blank while distilled water instead of sample was used for the control. The scavenging activity of DPPH radicals by the sample was calculated according to the following equation: DPPH radical scavenging activity (%) = $[1 - (A_{sample} - A_{blankl})/A_{control}] \times 100\%$, where A_{sample}, $A_{control}$, and A_{blank} were defined as absorbances of the sample, blank (without DPPH) and control (without EPS).

2.7.2. OH radical scavenging activity

The scavenging activity of hydroxyl radical was determined based on the method described by Zhao, Gui, Sun, Cao, and Zhang (2010). The varying concentrations of EPS (0.1–0.4 mg/ml, 1 ml) was incubated with a solution containing phenanthroline (7.5 mM, 1 ml), phosphate buffer (20 mM, pH 7.4, 0.5 ml), $FeSO_4$ (0.75 mM, 1.5 ml) and H_2O_2 (3%, 0.5 ml) at 37 °C for 1 h. The absorbance was measured at 536 nm using UV-2550 spectrophotometer (Shimadzu, Kyoto, Japan). The scavenging activity was calculated using the following equation: OH radical scavenging (or inhibition) rate (%) = $(A_{sample} - A_{blank})/(A_{control} - A_{blank}) \times 100\%$, where A_{sample}, $A_{control}$, and A_{blank} were defined as absorbances of the sample, control (without EPS) and blank (without H_2O_2 and EPS).

2.7.3. ABTS radical scavenging activity

TEAC (Trolox equivalent antioxidant capacity) assay measures the ability of a compound to eliminate or scavenge radicals compared with Trolox [(S)-(2)-6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid] as an antioxidant reference (Leung, Zhao, Ho, & Wu, 2009). The ABTS kit was bought from Biyutian Institute of Technology, China. Briefly, the ABTS radical cation solution was diluted with PBS (pH 7.4) to an absorbance (734 nm) of 0.7 ± 0.05 for assay. Ten microliters of the EPS solution was added to 200 μl of the diluted ABTS radical cation solution after 5 min the remaining amount of ABTS was determined at 734 nm using a spectrophotometer. The ABTS radical cation scavenging activity was expressed as Trolox equivalent antioxidant activity (TEAC) and the TEAC values of EPS samples were derived from the calibration curve generated with Trolox in the concentration range of 0–1.5 mM.

2.8. Evaluation of antitumor effect in vitro

For the antitumor effect study, human hepatoma (Hep G2) cells were cultured in DMEM medium supplemented with 15% heat-inactivated fetal bovine serum (FBS), penicillin (100 U/ml), and streptomycin (100 mg/l) in a humidified 5% CO_2 atmosphere at 37 °C (Chen, Zhao, Chen, & Li, 2008; Fan, Soccol, Pandey, & Soccol, 2007). The proliferation of Hep G2 was determined using the colorimetric MTT assay described by Mosmann (1983). Briefly, cells were seeded at a density of 2×10^4 cells/well in a 200 μl volume of the medium in 96-well plates and allowed to attach for 12 h. The dosages of crude EPS were 25, 50, 100, 200, and 400 μg/ml while the negative controls were treated with the medium. 10 μl MTT (0.4%) was added after 48 h. After incubated at 37 °C for 4 h, the supernatant was aspirated and then 100 μl DMSO was added to each well. Absorbance was measured at 490 nm by a 96 well microplate reader (Tecan, GENios ELIASA Co., Austria). All in vitro results were expressed as the inhibition ratio of tumor cell proliferation as follows: $[1 - (A_{sample} - A_{blank})/A_{control} - A_{blank}] \times 100\%$, where A_{sample}, $A_{control}$, and A_{blank} were defined as absorbances of the sample, control (without EPS) and blank (without cell). In order to verify whether the EPS did not decolorize the MTT, the absorbance was measured with and without the addition of EPS solution in MTT. There was no significant difference between MTT with buffer and MTT added EPS solution, which demonstrated that there was no such decolonization during assay.

2.9. Statistical analysis

Data were expressed as mean ± S.D. ($n = 2$ or $n = 3$). The statistical significance was determined by Student's *t*-test. Data Processing System (DPS Version 3.0) was used for the experimental designs and statistical analysis of the experimental data. The statistical differences were considered significant at $p < 0.05$.

Table 1
Effect of carbon, nitrogen and mineral source on mycelial growth and exopolysaccharides production by *F. trogii* in shake flask cultures.[a]

	Mycelial dry weight (g/l)	EPS production (g/l)	Final pH
Carbon (3%)			
Glucose	1.400 ± 0.1039	1.078 ± 0.1384	7.94
Fructose	0.827 ± 0.1172	1.214 ± 0.1358	7.02
Maltose	1.527 ± 0.0115	1.298 ± 0.1376	7.81
Sucrose	1.427 ± 0.0577	0.878 ± 0.1177	7.96
Soritol	1.540 ± 0.0283	0.177 ± 0.0487	7.97
Nitrogen (0.3%)			
Beef extract	3.230 ± 0.2715	1.442 ± 0.0513	5.36
Tryptone	3.250 ± 0.1088	1.531 ± 0.0093	4.63
Polyptone	4.170 ± 0.1131	1.463 ± 0.0189	4.98
Corn extract	4.850 ± 0.0424	1.116 ± 0.0283	4.60
Soy extract	11.60 ± 0.4808	0.988 ± 0.0256	4.87
Urea	2.490 ± 0.8191	0.819 ± 0.0535	8.06
Yeast extract	13.07 ± 0.0283	0.693 ± 0.0168	4.58
Peptone	6.560 ± 0.1099	0.723 ± 0.0439	3.98
Mineral sources (5 mM)			
Control	1.710 ± 0.2121	2.308 ± 0.3640	5.54
KH_2PO_4	2.600 ± 0.1131	3.454 ± 0.5047	4.69
NaCl	1.750 ± 0.0141	1.637 ± 0.1090	5.53
$MgSO_4$	1.960 ± 0.2263	2.495 ± 0.0419	5.59
$CaCl_2$	2.080 ± 0.1414	2.694 ± 0.3355	5.41
$MnCl_2$	1.093 ± 0.0643	2.682 ± 0.3020	5.31
$FeSO_4$	1.293 ± 0.1026	1.259 ± 0.3691	4.69

[a] Fermentation were carried out for 10 d at 25 °C with initial pH 5. Values are mean ± S.D. of replicated twice determinations.

3. Results and discussion

3.1. Effect of initial pH and temperature

In order to investigate the effect of initial pH on EPS production, *F. trogii* was cultivated in media with different initial pH (3.0–9.0) in shake flask cultures. The optimal pH for mycelial growth was 5.0, whereas the maximum EPS production was achieved at pH 6.0 (Fig. 1a). It is comparable that most of the enthomopathogenic fungi have pH optima within the range of 8–9 for EPS production (Bae et al., 2001; Bae, Sinha, Park, Song, & Yun, 2000; Park, Kim, Hwang, & Yun, 2001; Xu, Kim, Choi, Hwang, & Yun, 2003; Xu, Kim, Hwang, & Yun, 2006). To determine the optimal temperature for EPS production, this organism was cultivated at various temperatures. The optimal temperature was found to be 28 °C (Fig. 1b). In contrast, many kinds of mushrooms have relatively low temperature optimum, ranging from 20 to 25 °C (Bae et al., 2001; Xu et al., 2003, 2006).

3.2. Effect of carbon, nitrogen and mineral source

To find a suitable carbon source for EPS production in *F. trogii*, various carbon sources were provided at a concentration level of 30 g/l for 10 days in the basal medium. As shown in Table 1 among the carbon sources tested, the highest mycelial growth and EPS levels were obtained in maltose medium. In submerged cultures of enthomopathogenic fungi, different carbon sources, such as sucrose (Bae et al., 2001), glucose (Xu et al., 2003), and starch and maltose (Xu et al., 2006) have been reported for maximum EPS formation. From the practical point of view in the industry, maltose is a good medium ingredient considering its ease in use and low material cost compared to other nutrients.

To investigate the effect of nitrogen sources on EPS production, cells were cultivated in basal media containing various nitrogen sources at a concentration level of 3 g/l. Amongst 8 nitrogen sources examined, yeast extract was the most effective for mycelial growth whereas the maximum EPS production was achieved by trytone (Table 1). In comparison to organic nitrogen sources, inorganic

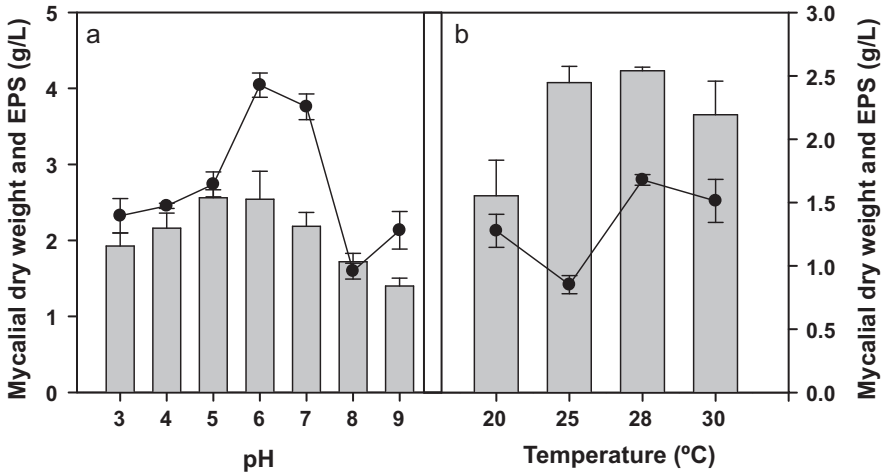

Fig. 1. Effect of initial pH (A) and temperature (B) on mycelial growth and exopolysaccharides production. Symbols: ( ) mycelial growth; (●) exopolysaccharides production. Fermentations were carried out for 10 d at agitation speed 150 rpm. Each value is mean of two independent experiments.

nitrogen sources gave rise to relatively lower mycelial growth and EPS production.

The influence of various mineral sources on EPS production was examined at a concentration level of 5 mM. Among the various mineral sources examined, KH_2PO_4 yielded good mycelial growth and EPS production and thus recognized as a favorable bioelement (Table 1). Similar observations were made by Kalogiannis, Lakovidou, Liakopoulou-Kyriakides, Kyriakidis, and Skaracis (2003) and Hwang, Kim, Xu, et al. (2003) in other mushroom fermentations. They suggested that K_2HPO_4 could improve EPS productivity through its buffering action, and essential phosphates were favorable for mycelial growth in submerged cultures of mushrooms.

3.3. Uniform design

To investigate the relationships between variables of the medium components and to optimize their concentrations for EPS production, the uniform design $U5(5^3)$ can be used. The uniform design was first proposed by Fang in 1978. Examples of successful applications of the uniform design method for improving technologies in various fields have been consistently reported since the 1980s. The uniform design has two characteristics: (1) the occurrence of each level of each factor in the experiments is once only; (2) The number of experimental trials equals the level of the number of factors. Uniform design tables can be expressed as $Un(t^s)$, where U stands for the uniform design, n for the number of experimental trials, t for the number of levels and s for the maximum number of factors. The advantages of the uniform design method lie in that the number of experiments is considerably less than that required by the other known experimental design techniques when the number of levels of factors is large, and it still develops usable results. According to preliminary experiments, we selected and varied five levels as shown in Table 2. The experimental conditions for each project are listed in Table 3, and the experimental results are also included in the last two columns of this table. The fermentation conditions of temperature, initial pH, agitation rate, and growth period were fixed to be 28 °C, 6.0, 150 rpm, and 10 d, respectively. The data obtained were interpreted using DPS 3.0.

Experimental designs resulted in a mathematical expression:

$$Y = K + aX_1 + bX_2 + cX_3 + \cdots + lX_1X_2 + mX_1X_3 + nX_2X_3 + \cdots$$

where Y is the dependent variable (yield), K is the constant representing the mean of the dependent variable obtained in each experiment, $X_1, X_2, X_3 \ldots$ are the independent variables, $X_1X_2, X_1X_3, X_2X_3 \ldots$, are the interaction terms and $a, b, c \ldots l, m, n \ldots$ are the coefficients. This expression describes the interaction of the different independent variables on the production of EPS. The two equations relate the coefficients obtained for EPS production to the experimental variables as follows:

$$Y_{EPS} = 0.0875833683 + 0.7166633309X_2 + 0.06104367757X_1 \times X_2 + 1.3091180553X_2 \times X_3$$
$$(R = 0.9995, \quad F = 331.8764, \quad P < 0.04).$$

To obtain high EPS production, the optimum compositions obtained by DPS were 5% sucrose, 0.5% yeast extract, and 8.87 mM K_2HPO_4. For testing the goodness of fit of the regression equation, the multiple coefficients of correlation R and F values were evaluated. The closer the value of R to 1, the better the correlation between the obtained and predicted values. The value of R is 0.9995

Table 2
Experimental factors and their levels for uniform design.

Factor	Levels				
	1[b]	2[b]	3[b]	4[b]	5[b]
X_1[a] (mM)	1	3	5	7	9
X_2[a] (%)	1	2	3	4	5
X_3[a] (%)	0.1	0.2	0.3	0.4	0.5

[a] Symbols X_1, X_2, and X_3 represent factors of K_2HPO_4, maltose and yeast extract.
[b] Symbols 1, 2, 3, 4, and 5 represent concentration levels of each factor.

Table 3
Application of uniform design $U5(5^3)$ to the mycelial growth and EPS production by *F. trogii*.[a]

Run no	Variables levels			Responses	
	X_1[b]	X_2[b]	X_3[b]	Dry cell weight (g/l)	EPS (g/l)
1[c]	1(1)	2(2)	4(0.4)	1.84 ± 0.14	2.64 ± 0.73
2[c]	2(3)	5(5)	2(0.2)	2.84 ± 0.42	5.96 ± 0.27
3[c]	3(5)	3(3)	1(0.1)	1.74 ± 0.33	3.46 ± 0.55
4[c]	4(7)	4(4)	5(0.5)	3.04 ± 0.34	7.27 ± 0.52
5[c]	5(9)	1(1)	3(0.3)	1.24 ± 0.09	1.83 ± 0.37

[a] Fermentation were carried out for 10 d at 28 °C with initial pH 6.
[b] The arrangements of column X_1, X_2, and X_3 were decided by uniform design for 3 (factor) × 6 (run number).
[c] Every row of run number represents one experimental replicate, every run was replicated twice.

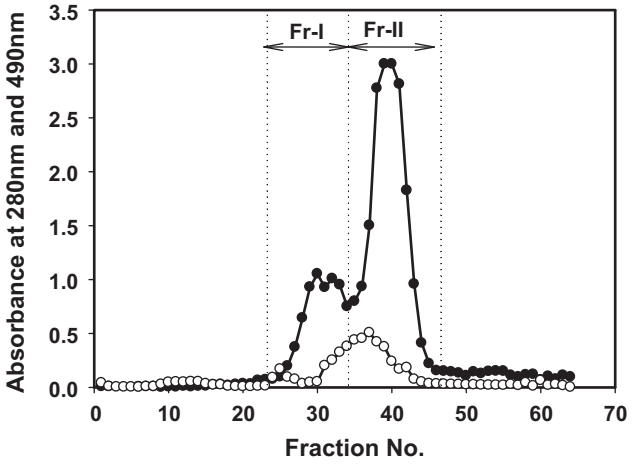

Fig. 2. Elution profiles of the polysaccharides (Fr-I and Fr-II) in Sepharose CL-6B chromatography. The volume of each fraction was 5 ml; elutes were analyzed by measuring the absorbance at 490 nm for carbohydrate (●) and the absorbance at 280 nm for protein (○).

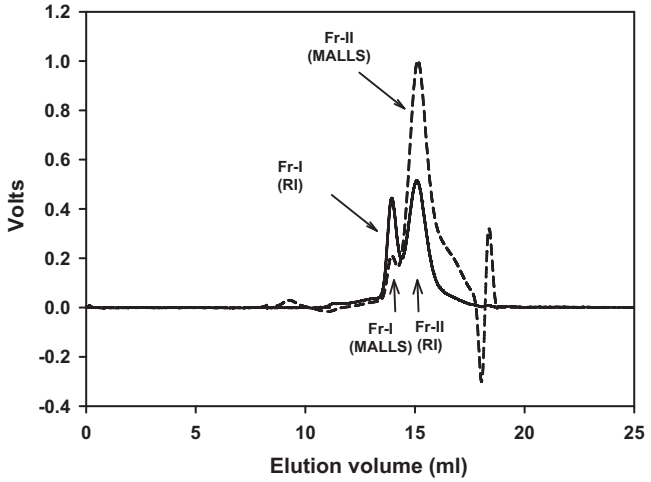

Fig. 3. Elution profiles of the polysaccharides (Fr-I and Fr-II) for the determination of molecular mass in SEC/MALLS system. For detailed analysis conditions, see Section 2. (–) MALLS detector, (—) refractive index detector. The peaks appearing at elution volume of around 17–19 ml is the baseline noise from the buffer solution.

for EPS, which indicates a good agreement between experimental and predicted values. The *F* value is a ratio of the mean square from the regression to the mean square from the real error. Generally, the calculated *F* value should be several times greater than the tabulated *F* value if the model is a good predictor of the experimental results and the estimated factor effects are real. Here, the computed *F* values of 331.8764 for EPS were greater than the tabulated $F_{4,1}$ within a rejection region having an *a*-level (axial distance; as this design is orthogonal) that is $p < 0.01$.

The maximum predicted value of EPS yield was 3.52 g/l, where the corresponding experimental responses were 3.04 g/l. The experimental and predicted values of EPS yields show good agreement with one another.

3.4. Purification and characterization of EPS with SEC/MALLS system

In gel filtration chromatography of the culture filtrate on Sepharose CL-6B, two fractions of EPSs (Fr-I and Fr-II), which consisted of polysaccharides and proteins, were coeluted as shown in Fig. 2. It was revealed that Fr-I and Fr-II were glycoproteins.

For the sake of comparison with the results of fractionation from Sepharose CL-6B chromatography with those of the MALLS detector, SEC coupled with MALLS and RI detectors was performed, and eventually the absolute molecular weights were determined for each EPS. SEC coupled with MALLS and RI detectors was performed, and eventually the absolute molecular weight peaks were determined for each Fr-I and Fr-II (Fig. 3), which was in good agreement with that of gel filtration chromatography on Sepharose CL-6B. Two peaks appeared between the elution volume of 14–15 and 15–17 ml, respectively. The molecular mass values for two eluted fractions were calculated for the portions of peaks, which lie within the peak ranges. The relevant molecular parameters of each EPS are summarized in Table 4. The weight average molar mass (M_w) of Fr-I and Fr-II were determined to be 1.007×10^5 and 2.393×10^4 g/mol, respectively. The low values of polydispersity ratio for four EPSs mean that these EPS molecules exist much less dispersed in aqueous solution without forming large aggregates (Hwang, Kim, Xu, et al., 2003). This information is important because the functional properties of polysaccharides can be greatly influenced by the molecular weight distribution. For each of these moments of the distribution, the root mean square (RMS) radii of the EPSs were calculated (Table 4). These data provide a measure of the EPS molecular size in terms of the RMS distance from the molecular center of gravity to its edge (Hwang, Kim, Choi, et al., 2003; Hwang, Kim, Xu, et al., 2003). The RMS radii for both peaks ranged from 9.7 to 10.8 nm with no clear trends (Table 4). The slope for Fr-I in the double logarithmic plots of RMS radius vs. molecular mass was shown in Table 4. The values of slope of Fr-I indicated 0.81, which implies that the Fr-I molecule exists as a rigid rod form in aqueous solution (see Section 2). However, since the RMS radii (9.7–9.9 nm) of Fr-I closed to 10 nm inferior limit of static light scattering, the DLS (Dynamic Light Scatting), X-ray Photoelectron Spectroscopy as well as Viscometer were used to further verify the molecular characteristics of Fr-I precisely (Coviello, Kajiwara, Burchard, Dentini, & Crescenzi, 1986; Thünemann, Knappe, Bienert, & Weidner, 2009). It also should be mentioned here that the slopes for Fr-II in the double logarithmic plots of RMS radius vs. molecular mass were not detected because the molecular weights of them were smaller than 5×10^4 g/mol in the aqueous system.

3.5. Antioxidant activity of F. trogii EPS

Antioxidant activities have been attributed to various reactions and mechanisms, such as radical scavenging, reductive capacity, prevention of chain initiation, and binding of transition metal ion

Table 4
Relevant molecular parameters of exopolysaccharides (Fr-I and Fr-II) and the double logarithmic plots of root mean square radius vs molecular mass for four groups exopolysaccharides of produced by submerged mycelial culture of *F. trogii* in MALLS analysis.

Parameters[a]	Fr-I (error %)	Fr-II (error %)
M_n[a] (g/mol)	9.697×10^4 (0.6)	2.071×10^4 (3)
M_w[a] (g/mol)	1.007×10^5 (0.5)	2.393×10^4 (2)
M_z[a] (g/mol)	1.037×10^5 (1.0)	2.608×10^4 (4)
M_w/M_n[b]	1.039 (0.8)	1.155 (4)
R_n[c] (nm)	9.7 (2.3)	n/a
R_w[c] (nm)	9.8 (2.2)	8.3 (13.0)
R_z[c] (nm)	9.9 (2.0)	10.8 (6.1)
Double logarithmic plots of root mean square radius vs molecular mass	0.81 (0.08)	n/a

[a] M_n, M_w, and M_z refer number-, weight-, *z*-average molecular weight, respectively.
[b] M_w/M_n means polydispersity ratio.
[c] R_n, R_w, and R_z refer number-, weight-, *z*-average square mean radius of gyration, respectively.

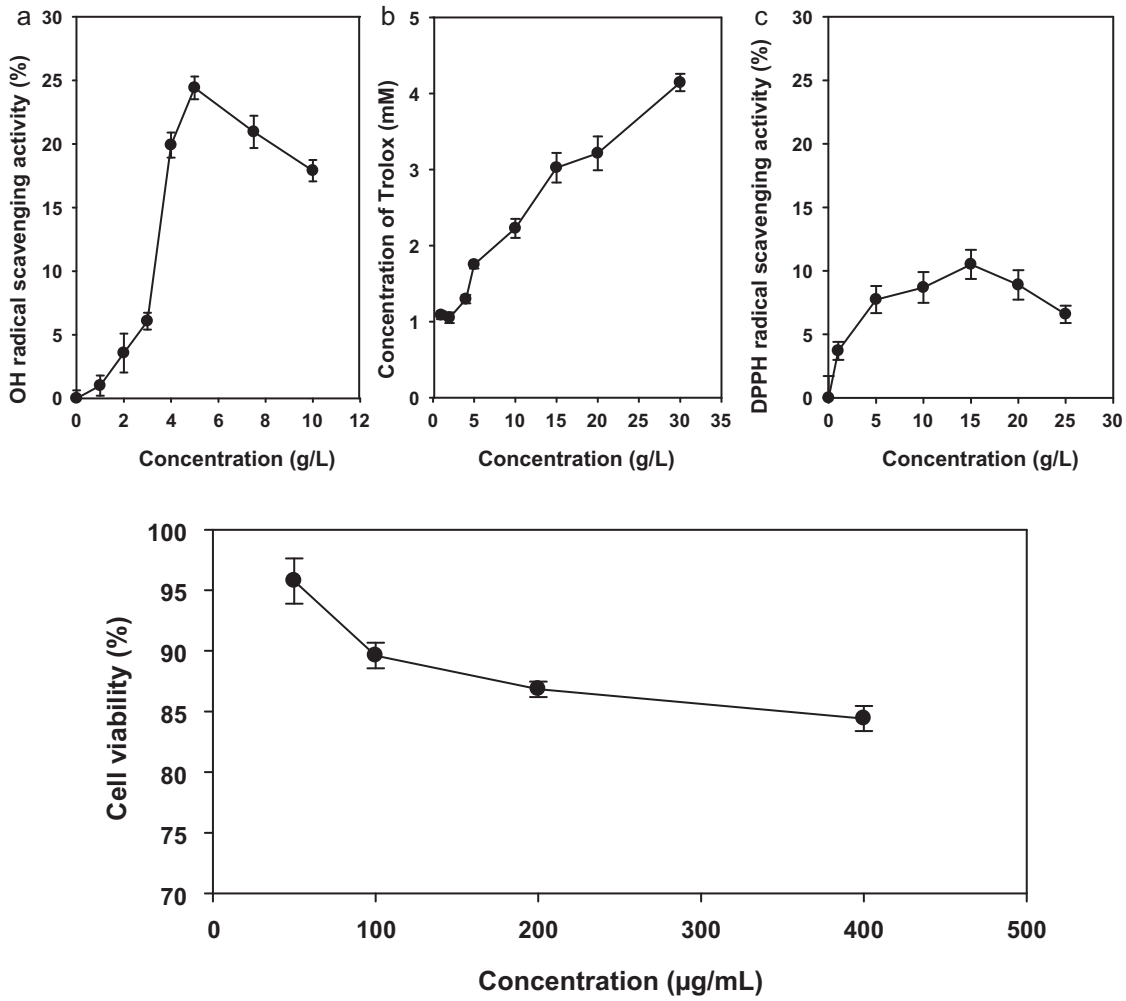

Fig. 4. Bioactivity of *F. trogii* polysaccharides. The results represent mean ± S.D. ($n = 3$). Upper figure: OH (A), ABTS (B) and DPPH (C) radical scavenging activity of *F. trogii* polysaccharides. Lower figure: Antitumor activity of *F. trogii* polysaccharides. HepG2 cells grown in 96-well plates and allowed to attach for 12 h. Cell viability was determined by MTT assay as described in the text.

catalysts (Frankel & Meyer, 2000). In this experiment, the in vitro antioxidant capacities of *F. trogii* EPS were evaluated using different biochemical methods including hydroxyl, DPPH and ABTS radical scavenging assay. Hydroxyl radical is the most reactive among reactive oxygen species (ROS) and it bears the shortest half-life compared with other ROS. Among the oxygen radicals, hydroxyl radical is the most reactive and induces severe damage to adjacent biomolecules (Sakanaka, Tachibana, & Okada, 2005). The results of hydroxyl radical scavenging assay are shown in Fig. 4a (upper figure). The OH radical scavenging rate of *F. trogii* EPS at 5 g/l was 24.4%. However, hydroxyl radical scavenging activity no longer increased when the concentration of EPS continued to increase. The TEAC of the EPS (Fig. 4b) also showed a steady increase with the concentration of EPS, and finally reached to 4.1 mM Trolox. However, the DPPH scavenging rates of EPS were not significant between 5 g/l and 20 g/l EPS ($p > 0.05$), the DPPH scavenging rate of EPS only reached to 10.5% (Fig. 4c). These results indicated that the EPS have potential antioxidant capacities.

3.6. Antitumor activities of F. trogii EPS

There is also increasing experimental evidence that regular incorporation of certain powdered medicinal mushrooms in the diets of animals or topical application of extracts can have a cancer prevention effect and restriction of tumor metastasis. Hep G2 cells are used as a model system for studies of liver metabolism because of their high degree of morphological and functional differentiation in vitro. Recently years, several EPS derived from macrofungi were employed for treatment of Hep G2 cells, and the studies showed that the EPS could induce cell apoptosis (Ikekawa, 2001; Wasser, 2002). Antitumor activities of *F. trogii* EPS are shown in Fig. 4 (lower figure). It could be found that the Hep G2 cell viability treated by *F. trogii* EPS decreased significantly from 95.8% to 84.4% with increasing concentration (50–400 μg/ml) of EPS ($p < 0.05$). The result showed that the use of *F. trogii* polysaccharides for treating Hep G2 cancer cells was limited. It could be due to the effective biological activities of EPS were not influenced by one single factor but combined other factors, such as solubility in water, branching frequency and forms.

4. Conclusions

An enthomopathogenic fungus, *F. trogii*, produced two groups of EPS during submerged cultures by optimized the culture conditions using uniform design, and the molecular characteristics were examined using a SEC/MALLS system. Furthermore, the bioactivities were investigated eventually. The data presented in this study clearly demonstrate the utility of the SEC/MALLS methodology for determining the absolute molecular weight and molecular dimensions (shapes) for two groups of EPS. It is expected that the MALLS

system can be widely applied to analyze the molecular characteristics for polysaccharides produced from either fruiting body or submerged cultures of mushrooms within a markedly short time. Static light scattering measures the average total scattering intensity over a selected time period. It provides a convenient method for deriving several molecular parameters simultaneously, including weight average molecular weight, RMS radii and other information. Of course, the molecular conformation is better to be verified with other techniques, such as DLS, X-ray Photoelectron Spectroscopy, Viscometer and AFM (atomic force microscopy) when the dimension of polysaccharide was closed to or fell below the reliable detection limit (10 nm) of the light scattering apparatus as showed in this work. The further studies on the relationship between their molecular properties and bioactivities are currently in progress in our laboratory. It is also necessary to establish the molecular-bioactivity relationship of the polysaccharides from *F. trogii* in order to expand its application.

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