Production Optimization, Molecular Characterization and Biological Activities of Exopolysaccharides from *Xylaria nigripes*

Y. P. Ma,[a] D. B. Mao,[b] L. J. Geng,[b] W. Y. Zhang,[b] Z. Wang,[b] and C. P. Xu[b]*

[a]China Tobacco Henan Industrial Co. Ltd, Zhengzhou, Henan, 450016, P.R.China

[b]College of Food and Biological Engineering, Zhengzhou University of Light Industry, Zhengzhou, Henan, 450002, P.R.China

Original scientific paper
Received: May 11, 2012
Accepted: January 14, 2013

The optimal culture conditions of exopolysaccharides (EPS) production in submerged culture medium by *Xylaria nigripes* were determined using orthogonal matrix method. The optimal medium (per liter) EPS was 60.0 g L^{-1} maltose, 1.0 g L^{-1} peptone, 5 mmol L^{-1} KH_2PO_4, and initial pH 7.0 at 28 ºC. In the optimal culture medium, the maximum EPS production was 11.967 g L^{-1} in shake flask. Two groups of EPSs (designated as Fr-I and Fr-II) were obtained from the culture filtrates by size exclusion chromatography (SEC), and their molecular characteristics were examined by a multiangle laser-light scattering (MALLS) and refractive index (RI) detector system. The weight-average molar masses of Fr-I and Fr-II of EPS were determined to be 6.327×10^4 and 1.478×10^4 g mol^{-1}, respectively. The SEC/MALLS analysis revealed that the molecular formation of Fr-I is of nearly globular shape. Furthermore, the experiments *in vitro* indicated that *X. nigripes* EPS exhibited high antioxidative effects though its antitumour activity was limited.

Key words:

Optimization, exopolysaccharides, *Xylaria nigripes*, molecular characterization, antioxidant, antitumour

Introduction

Modern pharmacological studies have shown that exopolysaccharides (EPS) produced from medicinal mushroom possesses anti-tumor, antioxidant, hypoglycemic activities, anti-radiation, hypolipidemic and hyperglycemic activities, anti-fatigue and other biological activities, which are also hotspots of research on functional factors of drugs and health food.[1–3] The submerged cultivation of medicinal mushroom has received great interest as a promising technique for efficient production of mycelia and exopolysaccharide (EPS).[4] Based on the chemically defined medium, the metabolic flux changes of EPS biosynthesis was clear to exploit. Meanwhile, polysaccharide obtained from submerged culture always exhibited similar biological activity as that of the polysaccharide extraction from fruiting body. [5]

Xylaria nigripes, known as *Wu Ling Shen*, is a high value medicinal fungus in traditional Chinese medicine, growing in the wild around abandoned termite nests. *X. nigripes*, especially the extract of sporocarp or mycelia, has been widely used in various applications including enhancement of immunity and hematopoiesis, treating of insomnia, trauma, as an anti-inflammatory, diuretic, and nerve tonic.[6–9] It also possesses good antioxidant and hepatoprotective activities.[10,11] However, to the best of our knowledge, the cultivation requirements of *X. nigripes* for exopolysaccharides production in submerged culture, the molecular characteristics as well as antioxidant and anti-tumor activities of the EPS have not been demonstrated.

Previous studies have indicated that polysaccharides bioactivity might be closely related to chemical properties and structural characteristics of polysaccharides.[12,13] Therefore, data of detailed molecular characterization of EPS as new safe compounds for functional foods or medicine has become a “hotspot” for research.

In this work, an optimum medium for EPS production of *X. nigripes* was investigated for the first time. The orthogonal matrix method was employed to determine the optimal level of each of the significant medium components in the medium. Furthermore, the EPSs fractions were isolated by gel filtration chromatography, and the molecular features were investigated by a SEC/MALLS system. Finally, antioxidant and antitumor activities of *X. nigripes* EPS *in vitro* were analyzed.

*Corresponding author: e-mail: c.p.xu@zzuli.edu.cn

Materials and methods

Microorganism and growth conditions

X. nigripes was kindly provided by Prof. Xinsheng He of the School of Life and Engineering, Southwest University of Science and Technology, Mianyang, Sichuan Province, China. It was originally collected from Shaozai county in Henan province, then authenticated and preserved at the Henan Province Microbiological Culture Collection Center (HPMCC no. 175624). Stock cultures were maintained on potato dextrose agar (PDA) slant. Slants were incubated at 26 °C for 8 days and stored at 4 °C, and sub-cultured every one month. The seed culture was grown in a 250 mL flask containing 50 mL of GP medium (0.3 % peptone, 3 % glucose, pH 6.5) at 26 ºC on a rotary shaker incubator (150 rpm) for 4 days. Flask culture experiments were performed in 250 mL flasks containing 50 mL media after inoculating with 4 % (v/v) of the seed culture.

Inoculum preparation

X. nigripes was initially grown on PDA medium in a Petri dish, and transferred into the seed medium by punching out 5 mm of the agar plate culture with a cutter of own design.[14]

Analytical methods

Samples collected from shake flasks were filtered through a membrane filter (0.45 μm, Millipore). The dry weight of mycelium was measured after repeated washing of the mycelial pellet with distilled water, and drying at 70 °C overnight to constant weight. The resulting culture filtrate was mixed with four times volume of absolute ethanol, stirred vigorously and kept overnight at 4 °C. The precipitated EPS was centrifuged at 11000 *g* for 15 minutes discarding the supernatant.[15] The precipitate of EPS was redissolved in distilled water, and the concentration of the EPS was determined by phenol-sulfuric acid method. To investigate the effect of initial pH on mycelial growth and the yield of EPS in shake flask cultures, the different initial pH was adjusted by addition of 1.0 M NaOH or HCl, and the temperature changes were conducted in the temperature-controlled shaking incubator. And each experiment was preformed tripartite.

Orthogonal matrix method

The orthogonal matrix $L_9(3^4)$ method can be used to investigate the relationships between variables of medium components, and optimize their concentrations for EPS production upon testing by the one-factor-at-a-time method. This enables us to determine which process variables affect the response. A logical next step is to determine the point in the important factors that leads to the best possible response. The three factors with varied three levels are shown in Table 1.

Table 1 – *Application of $L_9(3^4)$ orthogonal projects to the EPS production by X. nigripes*[*]

Variable	Levels		
	1	2	3
A, Maltose/(g L^{-1})	40	50	60
B, Peptone/(g L^{-1})	1	2	3
C, KH_2PO_4/(mmol L^{-1})	3	4	5

[*]Symbols A, B and C represent factors of maltose, peptone and KH_2PO_4. Symbols 1,2 and 3 represent concentration levels of each factor.

Purification of EPS

The ethanol precipitates of the crude EPS were treated with Seveg reagent (1:4 n-butanol/chloroform, v/v) to remove most proteins. After removing the proteins and Sevag reagent by centrifugation, the water phase was dialyzed against distilled water and lyophilized to yield polysaccharide. 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^{-1}. Protein concentration was determined according to the Bradford method[16] 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.[17] The protein moiety in the EPS was monitored by measuring the absorbance at 280 nm, whilst the carbohydrate moiety was monitored at 490 nm.

SEC/MALLS analysis

The molecular weights of the EPS were estimated by SEC coupled with MALLS Dawn DSP detector (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 0.1M PBS buffer (pH 6.8) containing 0.04 % diaminotetraacetic acid–disodium salt (Na_2EDTA) and 0.01 % sodium azide and filtered through 0.25 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^{-1}, while the injection volume and concentration was 100 μL and 2 mg mL^{-1}, 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,[18] in which the estimated dn/dc was 0.14 mL g^{-1}. 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.[19] 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:

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

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

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

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.[20]

Antioxidant activity tests

The antioxidant properties of EPS fractions were measured with three different assays as described below for more comprehensive evaluation of their activities against various free radicals (OH–, DPPH and ABTS radicals). In these following assays the EPS samples were predissolved in water and tested at various concentrations in parallel with vitamin C (Vc) as an antioxidant reference (positive control) (or Trolox as a reference in the TEAC assay).

The scavenging activity of hydroxyl radical was determined based on the method described by Zhao *et al*.[21] The varying concentrations of polysaccharides (2–10 mg mL^{-1}, 2 mL) were incubated with a solution containing phenanthroline (7.5 mmol L^{-1}, 1 mL), phosphate buffer (50 mmol L^{-1}, pH 7.4, 1 mL), $FeSO_4$ (3.25 mmol L^{-1}, 1 mL) and H_2O_2 (3 %, 0.5 mL) at 37 °C for 1 h. The resulting sample was filtered through a Whatman filter paper No.2 (Whatman International Ltd, Maidstone, UK). The absorbance was measured at 510 nm using UV-2550 spectrophotometer (Shimadzu, Kyoto, Japan). The scavenging activity was calculated using the following equation:

$$\text{OH radical scavenging rate (\%)} = \\ = (A_{sample}\text{-}A_{blank})/(A_{control}\text{-}A_{blank}) \times 100\ \% . \quad (1)$$

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).

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 *et al*..[22] The different amount of EPS was dissolved in distilled water to make different concentration solutions. Two millilitres of 0.1 g L^{-1} DPPH in 50 % ethanol was added to 2 mL of the EPS solution. The absorbance was measured at 517 nm after 20 minutes of incubation at 25 °C. Fifty-percent ethanol instead of DPPH was used for the blank, while distilled water instead of sample was used for control. The scavenging activity of DPPH radicals by the sample was calculated according to the following equation:

$$\text{DPPH radical scavenging activity (\%)} = \\ = [1 - (A_{sample} - A_{blankl})/A_{control}] \times 100\ \% . \quad (2)$$

Where A_{sample}, $A_{control}$, and A_{blank} were defined as absorbances of the sample, blank (without DPPH) and control (without EPS).

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-tetramethyl-chroman-2-carboxylic acid] as an antioxidant reference.[23] The ABTS kit was bought from Biyutian Institute of Technology, China. Briefly, the ABTS radical cation solution was diluted with ethanol or PBS (pH 7.4) to an absorbance (734 nm) of 0.7±0.05 for assay. Ten microlitres of the exopolysaccharides solution was added to 200 μL of the diluted ABTS radical cation solution, after 5 minutes 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 mmol L^{-1}.

Evaluation of antitumour effect in vitro

For the antitumour 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^{-1}), and streptomycin (100 mg L^{-1}) in a humidified 5 %

CO_2 atmosphere at 37 °C.[24] The proliferation of Hep G2 was determined using the colorimetric MTT assay described by Mosmann.[25] Briefly, the 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 polysaccharides were 25, 50, 100, 200, 400 μg mL^{-1} while the negative controls were treated with the medium. 10 μL MTT (0.4 %) was added after 48 h. After incubation 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). Since doxorubicin is considered to be one of the most effective anticancer agents currently used, this was used as the positive control to validate experimental systems in vitro. All in vitro results were expressed as the inhibition ratio of tumor cell proliferation as follows:

$$\text{Antitumour activity (\%)} = \\ = [1 - (A_{sample} - A_{blank}) / (A_{control} - A_{blank}] \times 100\ \%. \quad (3)$$

Where A_{sample}, $A_{control}$, and A_{blank} were defined as absorbances of the sample, control (without EPS) and blank (without cell).

Statistical analysis

Data were expressed as mean ± S.D. (n = 3). The statistical significance was determined by Student's t-test. Experimental results from the experimental design were statistically subjected to analysis of variance (ANOVA) using SPSS (version 11.0, Chicago, IL). Probability values < 0.05 and < 0.01 were regarded as statistically significant and highly significant, respectively.

Results and discussion

Effect of initial pH and temperature

In order to investigate the effect of initial pH on mycelial growth and EPS production, *X. nigripes* was cultivated in the GP medium with different initial pH (3.0–9.0) in shake flask cultures. Maximum EPS concentration (4.29 g L^{-1}) was obtained at an initial pH 7.0 though the maximum biomass concentration (3.32 g L^{-1}) was investigated at an initial pH of 4.0 (Fig. 1A). It is interesting to note that many kinds of fungi have acidic pH optima for EPS production during submerged culture.[26] To find the optimal temperature, this organism was cultivated at various temperatures, where the optimum temperature was found to be 28 °C for EPS production (Fig. 1B).

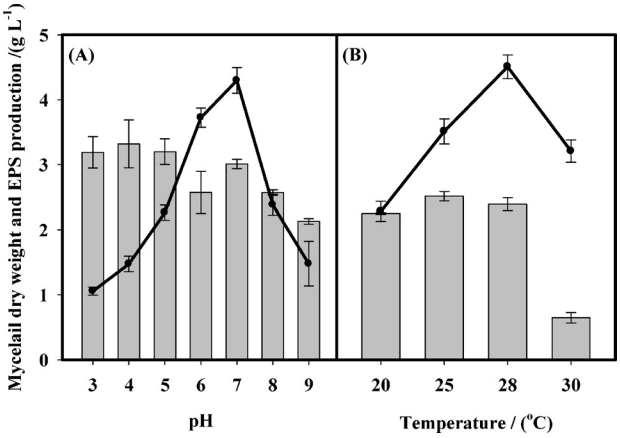

Fig. 1 – *Effect of pH and temperature on mycelial growth and EPS production by X. nigripes in shake flask cultures. Symbols: (■) mycelial growth; (●) exopolysaccharides production*

Screening of medium constituents for EPS production

The one-factor-and-a-time method was used to find the optimal nitrogen, carbon and mineral sources for EPS production. The influence of nitrogen sources on EPS production was examined by various nitrogen sources instead of peptone at the concentration level of 3 g L^{-1} in the GP medium. Among the various nitrogen sources examined, peptone yielded the maximum EPS production, although the highest mycelial growth was achieved when yeast extract was employed (Fig. 2A). Those results indicated that the nutritional requirement for EPS production in *X. nigripes* was not always consistent with that of mycelial biomass.

To investigate the effect of carbon sources for the EPS production, cells were cultivated in the medium containing various carbon sources. Each carbon source was added to the GP medium at a concentration level of 30 g L^{-1}. Amongst carbon sources examined, maltose was favorable for the EPS production (Fig. 2B).

To find a suitable mineral source for EPS production in *X. nigripes*, various mineral sources were provided at a concentration level of 5 mmol L^{-1} in the GP medium. Among the various mineral sources examined, KH_2PO_4 yielded maximum EPS production (Fig. 2C).

Optimization of screened medium constituents by orthogonal matrix method

Based on the results of one-factor-at-a time method, where the variables (maltose, peptone and KH_2PO_4) determined as the optimal carbon, nitrogen and mineral sources for EPS production, the orthogonal matrix method was employed to optimize

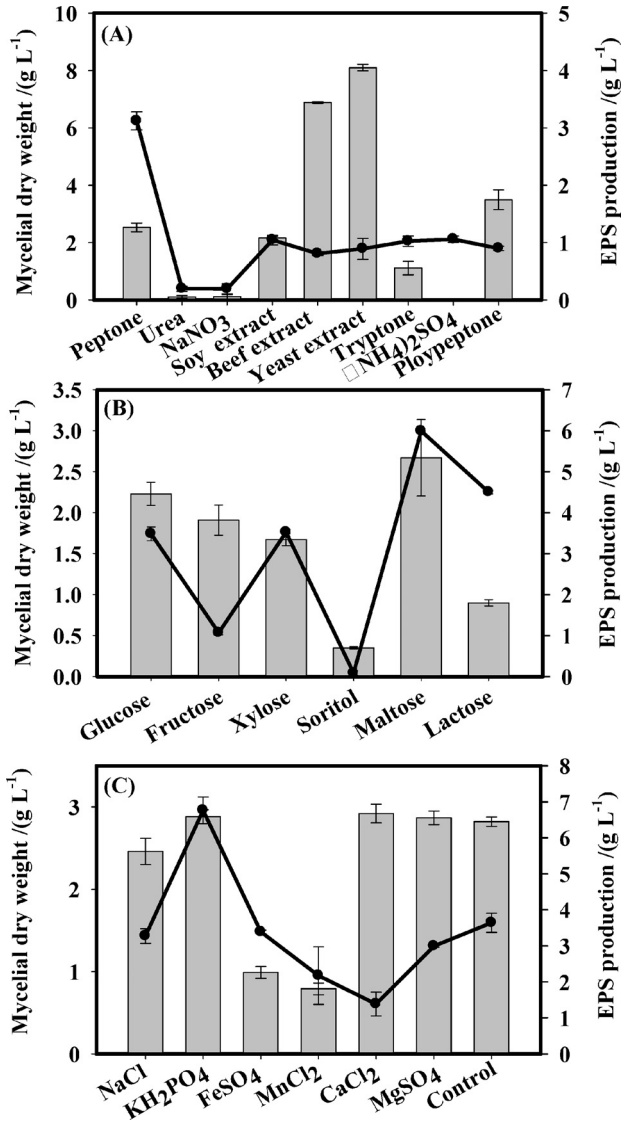

Fig. 2 – *Effect of nitrogen, carbon and mineral source on mycelial growth and EPS production by X. nigripes in shake flask cultures. Symbols: (■) mycelial growth; (●) exopolysaccharides production. Fermentations were carried out for 8 d at agitation speed 150 rpm. Each value is mean of three independent experiments*

Table 2 – *Analysis of media on EPS production by X. nigripes in shake flask cultures with Orthogonal Projects*[*]

Runs	Level of maltose, A	Level of peptone, B	Level of KH_2PO_4,C	EPS production (g L^{-1})
1	1	1	1	6.632±0.3187
2	1	2	2	6.751±0.3187
3	1	3	3	7.451±0.0183
4	2	1	3	6.419±1.0233
5	2	2	1	3.773±1.8789
6	2	3	2	5.482±0.8220
7	3	1	2	11.056±1.7195
8	3	2	3	9.829±0.0754
9	3	3	1	7.356±1.6104
K_1	6.943[a]	8.037	7.313	
K_2	5.223	6.783	6.843	
K_3	9.417	6.763	7.427	
R	4.194[b]	1.274	0.584	

[*]Fermentation was carried out for 8 d at 28 °C with initial pH 7.0. The arrangements of column A, B and C were decided by orthogonal design; every row of run number represents one experimental triplicate.
[a]$k_i^A = \Sigma$ mycelial yield at $A_i/3$
[b]$R_i^A = \max\{K_i^A\} - \min\{K_i^A\}$.

Table 3 – *Analysis of variance (ANOVA) of the orthogonal matrix method for optimization of EPS production by X. nigripes*

Source	Type Sum of squares	*df*	Mean Squares	*F*-value	Sig.
A	26.603	2	13.302	46.027	0.021
B	3.186	2	1.593	5.512	0.154
C	7.330	2	3.665	12.681	0.073
Error	0.578	2	0.289		
Total	503.518	9			
Corrected Total	37.697	8			

their individual concentration. The experimental conditions for each project are listed in Table 2, and experimental results are also included in the last two columns of this table. According to the order of magnitude of R value (maximum difference) as shown in Table 2, the order of effects of all factors on EPS production was maltose > peptone > KH_2PO_4. The corresponding ANOVA for EPS production is presented in Table 3. It was clearly observed that maltose had significant effects on the EPS production ($P<0.05$). This result pointed out that the effect of maltose was more important than that of other nutrients. In terms of the maximum K value of each factor, optimal combination for EPS was 60.0 g L^{-1} maltose, 1.0 g L^{-1} peptone, 5 mmol L^{-1} KH_2PO_4. The results indicated that a relatively high C: N ratio was desired for EPS biosynthesis, which was in accordance with previous reports.[27,28]

In order to confirm the optimization results, the suggested medium components were confirmed in triplicate. The maximum predicted value of EPS yield obtained was 12.81 g L^{-1} where corresponding experimental response was 11.967 g L^{-1}. The experimental and predicted values of EPS yields show good agreement with one another.

Purification and characterization of EPS

In gel filtration chromatography of the culture filtrate on Sepharose CL-6B, two fractions of EPSs (Fr-I and Fr-II) were coeluted as shown in Fig. 3. It might be revealed that Fr-II was glycoprotein, although further investigation by electrophoresis or other chromatography is needed. It should be noted that there are significant areas of overlap between the two fractions and further purification process is necessary to investigate the detailed chemical and biological characterization of the individual fraction. However, the SEC/MALLS approach could be useful in providing major insight into the characterization of biopolymers without carrying out elaborate fraction procedures prior to analysis.[29] A typical chromatogram with MALLS and RI detection is shown in Fig. 4, which

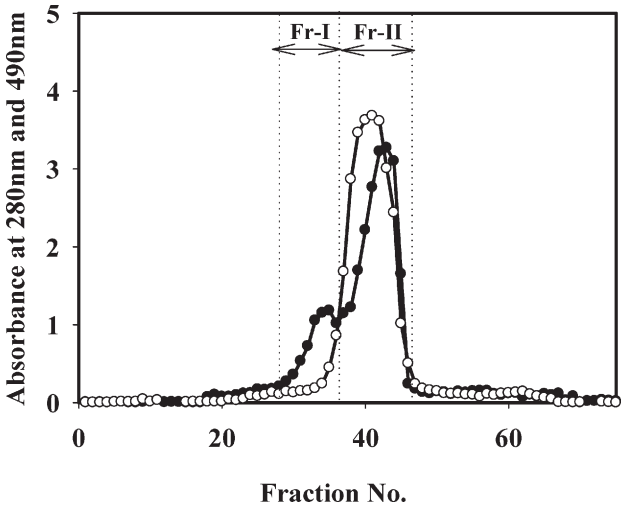

Fig. 3 – *Elution profiles of the polysaccharides (Fr-I and Fr-II) in Sepharose CL-6B chromatography. Elutes were analyzed by measuring the absorbance at 490 nm for carbohydrate (●) and the absorbance at 280 nm for protein*

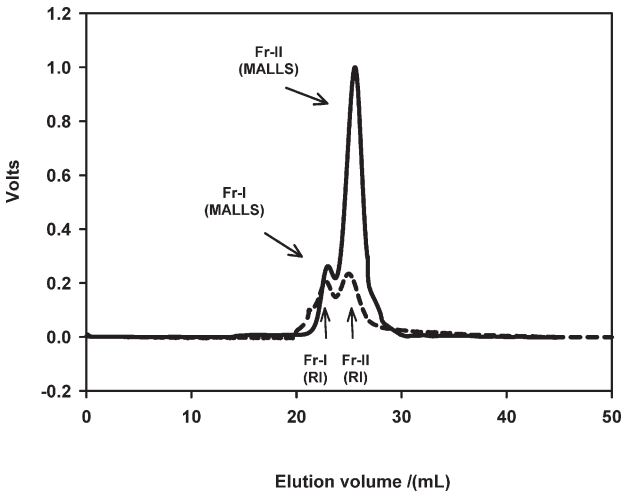

Fig. 4 – *Elution profiles of the polysaccharides (Fr-I and Fr-II) for the determination of molecular mass in SEC/MALLS system. For detailed analysis conditions, see Materials and methods. (– –) MALLS detector, (—) refractive index detector*

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 two groups exopolysaccharides of produced by submerged culture of X. nigripes in MALLS analysis*

Parameters*	Fr-I (error %)	Fr-II (error %)
M_n/(g mol^{-1})	4.848×10^4 (0.5)	1.30×10^4 (1)
M_w/(g mol^{-1})	6.327×10^4 (0.6)	1.478×10^4 (1)
M_z/(g mol^{-1})	1.302×10^5 (2)	1.763×10^4 (2)
M_w/M_n	1.305 (0.8)	1.137 (1)
R_n/(nm)	30.3 (2)	31.8 (4)
R_w/(nm)	31.5 (2)	32.5 (4)
R_z/(nm)	35.7 (2)	32.9 (4)
double logarithmic plots of root mean square radius vs molecular mass	0.21 (0.05)	n/a

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

was in good agreement with that of gel filtration chromatography on Sepharose CL-6B. Fr-I and Fr-II peaks appeared between the elution volume of 20.0–23.8 and 23.8–29.4 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 (Mw) of Fr-I and Fr-II were determined to be 6.327×10^4 and 1.478×10^4 g mol^{-1}, respectively. *M*w/*M*n (the polydispersity), is a measure of the width of molecular mass distribution. The low values of polydispersity ratio for all EPSs mean that these EPS molecules exist much less dispersed in aqueous solution without forming large aggregates.[30] The RMS radii for both peaks ranged from 30.3 to 35.7 nm with no clear trends. The slope for Fr-I in the double logarithmic plots of RMS radius versus molecular mass is shown in Table 4. The values of the slope of Fr-I indicated 0.21, which implies that the Fr-I molecule exists in nearly globular shape in aqueous solution (see SEC/MALLS analysis). It should be mentioned here that the slope for Fr-II in the double logarithmic plots of RMS radius versus molecular mass was not detected because the molecular weights were smaller than 5×10^4 g mol^{-1} in the aqueous system.

Antioxidant activity of X. nigripes polysaccharides

The mushroom EPSs have been demonstrated to play an important role as free radical scavengers in the prevention of oxidative damage in living organisms and can be explored as novel potential an-

tioxidants. Moreover, previous studies have indicated that polysaccharides antioxidant activity might be closely related to chemical properties and structural characteristics of polysaccharides.[31] 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 catalysts.[32] In this experiment, the *in vitro* antioxidant capacities of *X. nigripes* 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 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.[33] The results of hydroxyl radical scavenging assay are shown in Fig. 5A. The OH^- scavenging rate of *X. nigripes* EPS showed a steady increase with the concentration of EPS, and finally reached 87.19 % at the concentration of 10 mg mL^{-1} (closed to scavenging activity of vitamin C). The similar trends were observed DPPH and ABTS radical scavenging. The TEAC of the EPS was up to 3.92 mmol L^{-1} Trolox at the concentration of 4 mg mL^{-1} (Fig. 5B). And the DPPH scavenging rate of EPS at 3.0 mg mL^{-1} reached 27.80 % (Fig. 5C), although it is still quite lower than that of Vitamin C. These results indicated that EPSs have potential antioxidant capacities. Ko *et al*.[9] also pointed out that the ethanol extract from *X. nigripes* mycelia possessed potent antioxidant and antiradical activities. Sun *et al*. (2009) showed that the low-molecular-weight (6.55×10^3 g mol^{-1}) fragments of EPS had stronger antioxidant activity than the high-molecular-weight fragments (6.06×10^4 g mol^{-1} and 2.56×10^5 g mol^{-1}) extracted from *Porphyridium cruentum*.[34] However, the antioxidant mechanism of *X. nigripes* polysaccharides is not fully clear as yet, and it is an absolute necessity for the development of the correlation between the chemical characteristics and the antioxidant property of polysaccharide isolated from submerged culture of *X. nigripes* for further investigation. It is well-known that the bioactivities of polysaccharides can be affected by many factors including chemical components, molecular mass, structure, conformation, even the extraction and isolation methods.[12]

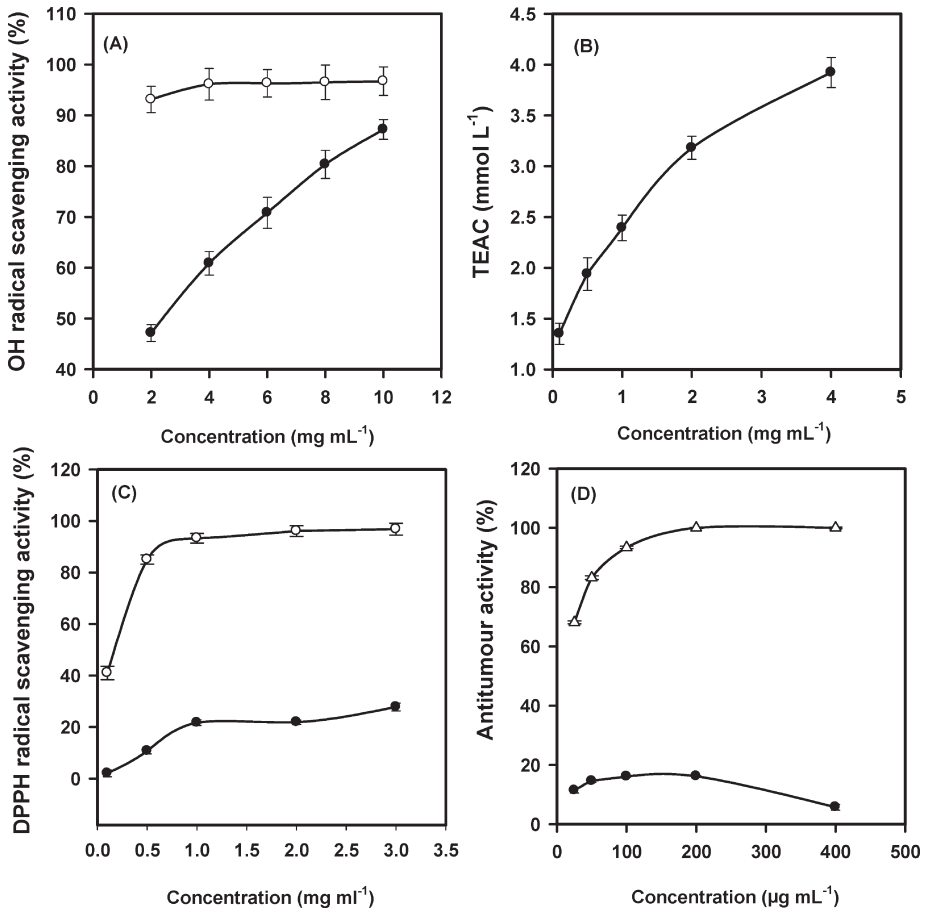

Fig. 5 – *Bioactivity of X. nigripes polysaccharides. The results represent mean ± S.D. (n = 3).OH (A), ABTS (B) and DPPH (C) radical scavenging activity and antitumour activity (D) of X. nigripes polysaccharides. Symbols: (●) EPS; (○) Vitamin C; Δ Doxorubicin. HepG2 cells grown in 96-well plates and allowed to attach for 12 h. Cell viability was determined by MTT assay as described in the text*

Antitumour activities of X. nigripes polysaccharides

Recently, many polysaccharides and polysaccharide-protein complexes have attracted more attention in the biochemical and medical areas due to their antiproliferative effects on cancer cells.[4,35] The molecular weight is very important to antitumor activity of the mushroom EPS. It has been reported that EPS from the mycelia of *Pleurotus tuber-regium* showed relatively higher antitumour activity in the M_w range 40 to 80 $\times 10^4$ than other fractions M_w below 5 $\times 10^4$ *in vivo* and *in vitro*.[12] The biological activities were closely related with solubility in water, molecular size, branching frequency and forms. In this work, to evaluate the effect of growth inhibition, various concentrations of *X. nigripes* EPS (50 μg mL^{-1} –400 μg mL^{-1}) were added to culture medium of human hepatocellular carcinoma Hep G2 cells. It could be found that antitumour activities of *X. nigripes* EPS reached 16.08 % at the concentration of 100 μg mL^{-1} (Fig. 5D). However, the positive control doxorubicin exhibited a higher antitumour activity toward Hep G2 cancer cells than *X. nigripes* EPS. This result showed that the use of *X. nigripes* polysaccharides for treating Hep G2 cancer cells was limited.

Conclusions

In this work, *X. nigripes* optimized submerged culture conditions for EPS production were addressed with the orthogonal matrix method. Two groups of EPSs were obtained by gel filtration chromatography and characterized by SEC/MALLS analysis. The SEC/MALLS approach was expected to provide greater insight into the conformation and component of biopolymers with high polydispersity without further isolation and purification. Furthermore, the antioxidant and antitumour capacities of EPSs were determined. The biological activity of polysaccharides is usually influenced by various molecular characterizations. Therefore, further research is ongoing in our laboratory to elucidate the complete structure and bioactivity of individual EPS fractions.

References

1. *Chihara, G., Hamuro, J., Maeda, Y., Arai, Y.,* Fukuoka, F., Cancer Res. **30** (1970) 2776.
2. *Wasser, S. P.,* Appl. Microbiol. Biotechnol. **60** (2002) 25.
3. *Zheng, W., Zhao, Y., Zheng, X., Liu, Y., Pan, S., Dai, Y., Liu, F.,* Appl. Microbiol. Biotechnol. **89** (2011) 157.
4. *Wasser, S.,* Appl. Microbiol. Biot. **60** (2002) 258.
5. *Tang, Y. J., Zhu, L. W., Li, H. M., Li D. S.,* Food Technol. Biotechnol. **45** (2007) 221.
6. *Xu, J. T.*, Chinese medicinal mycology. Beijing Medical University and Peking Union Medical College Press, Beijing (Ed.), pp 664–675, (in Chinese), 1997
7. *Ma, Z. Z., Zuo, P. P., Chen, W. R., Tang, L. J., Shen, Q., Sun, X. H., Shen, Y.,* Chinese Pharm. J. **34** (1999) 374.
8. *Dai, Y. C., Yang, Z. L.,* Mycosys-tema **27** (2008) 801 (in Chinese)
9. *Ko, H. J., Song, A., Lai, M. N., Ng, L. T.,* Am. J. Chinese Med. **37** (2009) 815.
10. *Wu, G.,* Acta Microbiologica Sinica **41** (2001) 363.
11. *Song, A. R., Ko, H. J., Lai, M. N., Ng, L. T.,* Immunopharm. Immunot. **33** (2011) 454.
12. *Zhang, M., Zhang, L., Cheung, P. C. K., Ooi, V. E. C.,* Carbohyd. Polym. **56** (2004) 123.
13. *Chen, H. X., Zhang, M., Qu, Z. S., Xie, B. J.*, Food Chem. **106** (2008) 559.
14. *Park, J. P., Kim, S. W., Hwang, H. J., Yun, J. W.,* Lett. Appl. Microbiol. **33** (2001) 76.
15. *Bae, J. T., Sinha, J., Park, J. P., Song, C. H., Yun, J. W.,* J. Microbiol. Biotechnol. **10** (2000) 482.
16. *Bradford, M. M.*, A rapid and sensitive method for the quantization of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72** (1976) 248–254.
17. *Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A., Smith, F.,* Anal. Chem. **28** (1956) 350.
18. *Jumel, K., Fiebrig, I., Harding, S. E.,* Int. J. Biol. Macromol. **18** (1996) 133.
19. *Wyatt, P. J.*, Anal. Chim. Acta **272** (1993) 1.
20. *Lim, J. M., Joo, J. H., Kim, H. O., Kim, H. M., Kim, S. W., Hwang, H. J., Yun, J. W.,* Carbohyd. Polym. **61** (2005) 296.
21. *Zhao, J., Gui, L. N., Sun, J. M., Cao, Y., Zhang, H.,* J. Life Sci. **4** (2010) 17.
22. *Eloff, J. N., Katerere, D. R., McGaw, L. J.,* J. Ethnopharmacol. **119** (2008) 686.
23. *Leung, P. H., Zhao, S. N., Ho, K. P., Wu, J. Y.,* Food Chem. **114** (2009) 1251.
24. *Chen, W., Zhao, Z., Chen, S., Li, Y.,* Bioresour. Technol. **99** (2008) 3187.
25. *Mosmann, T.,* J. Immunol. Methods **65** (1983) 55.
26. *Fang, Q., Zhong, J.,* Process Biochem. **37** (2002) 769.
27. *Kim, S. W., Hwang, H. J., Xu, C. P., Sung, J. M., Choi, J. W., Yun, J. W.,* J. Appl. Microbiol. **94** (2003) 120.
28. *Somasheker, D., Joseph, R.,* World J. Microbiol. Biotechnol. **16** (2000) 491.
29. *Li, Y., Weiss IV, W. F., Roberts, C. J.,* J. Pharm. Sci. **98** (2009) 3997.
30. *Hwang, H. J., Kim, S. W., Xu, C. P., Choi, J. W., Yun, J. W.,* J. Appl. Microbiol. **94** (2003) 708.
31. *Wang, Z. J., Luo, D. H.*, Carbohyd. Polym. **68** (2007) 54.
32. *Frankel, E. N., Meyer, A. S.,* J. Sci. Food Agric. **80** (2000) 1925.
33. *Sakanaka, S., Tachibana, Y., Okada, Y.,* Food Chem. **89** (2005) 569.
34. *Sun, L., Wang, C., Shi, Q., Ma, C.,* Int. J. Biol. Macromol. **45** (2009) 42.
35. *Peng, Y., Zhang, L., Zeng, F., Kennedy, J.K.,* Carbohyd. Polym. **59** (2004) 385.