Production, structural characterization, and antiproliferative activity of exopolysaccharide produced by *Scleroderma areolatum* Ehrenb with different carbon source

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Abstract

The effects of different three carbon sources, that is, glucose, fructose, and sucrose, on production, molecular properties and antiproliferative activity of exopolysaccharide (EPS), were evaluated in the submerged culture of *Scleroderma areolatum* Ehrenb. Among carbon sources examined, the addition of sucrose maximizes the mycelia production, while fructose could maximize the EPS yield. Although the predominant carbohydrate compositions identified were gluconic acid and mannose, the monosaccharide composition of EPSs was also different significantly. FT-IR spectral analysis revealed there was no significant difference among the prominent characteristic groups in three EPSs. The molecular weight of EPSs was also affected by carbon source, being generally lower compared with that with glucose. However, all EPSs molecule existed as nearly globular shape form in aqueous solution. The variation of carbon sources also affected antiproliferative activity examined in vitro using cell proliferation assay. Fructose was optimal carbon source giving higher antiproliferative activity probably due to the relatively high contents of xylose in the EPS with low molecular weight.

Keywords *Scleroderma areolatum* Ehrenb · Exopolysaccharide · Carbon source · Antiproliferative activity · Molecular weight

Introduction

Scleroderma areolatum Ehrenb is a basidiomycete fungus and a member of the genus *Scleroderma* [1]. Studies have shown that the bioactive metabolites isolated from fruiting bodies of *Scleroderma* were observed to have antimicrobial, anti-inflammatory, antitussive, hemostatic, and insecticidal antiulcer activities [2, 3]. Moreover, the dibromo derivative and acetate derivative isolated from *Scleroderma citrinum* demonstrated cytotoxic activity. [4]

Currently, much interest is focused on the exopolysaccharides (EPS) produced by submerged culture of medicinal fungi due to their many biological activities, such as antiproliferative, immunomodulatory, and anti-tumor activities [5, 6]. The EPS obtained from the submerged culture is easier than the internal-polysaccharide in the mycelium and exhibits similar biological activity, and therefore, many researchers have attempted to obtain EPS by submerged culture of fungi [7, 8]. Carbon source is one of the most important nutrients for EPS production and may be utilized by the fungal cells and converted to uridine-diphosphate (UDP)-monosaccharides for EPS synthesis [9, 10]. It is demonstrated that the carbon source clearly affects the yields and compositions of the EPS [11]. However, to the best of our knowledge, the effect of different carbon sources on EPS production, chemical characterization, and molecular property, especially antiproliferative activity of EPS was seldom reported. In this study, the effect of the carbon sources, glucose, fructose, and sucrose, on molecular features and antiproliferative potential of *S. areolatum* Ehrenb EPS by submerged cultivation was investigated.

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Material and methods

Chemicals

The carbon sources (glucose, fructose, and sucrose) and chemicals for medium culture were of reagent grade, while other chemicals were of analytical grade.

Fungi and growth conditions

Scleroderma areolatum Ehrenb was previously isolated from Wanda Mountains of Heilongjiang province and kept in the Henan Province Microbiological Culture Collection Center (HPMCC3564865). Stock cultures were maintained on potato dextrose agar (PDA) slants. Growth experiments were performed using different carbon source according to the following procedure. Firstly, *S. areolatum* Ehrenb was transferred onto a petri dish with the media of 40 g/L glucose, 4 g/L malt extract, and 20 g/L agar by punching out 5 mm of the agar plate culture with a cutter and cultivated for 8 days at 26 °C. The fungus was subcultured on each carbon source at least twice just prior to experimental use. Seed cultures on the flask were obtained 250 ml Erlenmeyer flasks filled with 50 mL of media (40 g/L glucose, 4 g/L malt extract, and 4 mmol/L KH_2PO_4 , pH = 7). Fermentation medium was inoculated with 4% (v/v) liquid 4-day old seed culture and incubated at 28 °C in a 5 L stirred tank (Infors, Switzerland). The fermentation was carried out in the same medium as the seed culture under the conditions of a temperature of 28 °C, aeration rate of 2 vvm, stirring speed of 160 rpm, pH of 7, working volume of 3 L, and culture time of 8 days. To investigate the effects of different carbon sources on EPS production and chemical structure, glucose in agar plates and submerged media was replaced by fructose or sucrose. All experiments were performed in triplicate, and the results were represented by mean $\pm$ S.D. (standard deviation).

Determination of EPS quantification and mycelia biomass

The culture broth was centrifuged at 9000 rpm for 15 min to separate the biomass from the medium. The precipitated biomass was rinsed three times with distilled water and dried at 70 °C to a constant weight [12]. To collect crude EPS, the resulting supernatant was mixed with four volumes of pure ethanol. The precipitated EPS was collected by centrifugation at 9000 rpm for 15 min and redissolved in distilled water. And then the protein was removed by Sevag reagent (1:4 n-butanol/chloroform, v/v). After removing the proteins, the aqueous phase was dialysis in distilled water and freeze-dried to obtain the crude EPS. The total EPS in the culture medium was determined by the phenol–sulfuric acid method, using glucose as the standard [13].

Purification of EPS

The crude EPS was purified by gel filtration chromatography on Sepharose CL-6B column (2.4 cm $\times$ 100 cm, Sigma Chemical Co., St Louis, MO) using 0.2 M NaCl solution as elution buffer at a flow rate of 0.6 ml/min. Fractions (5.0 ml/tube) were collected by an automatic collector. The total carbohydrate content in the EPS was also determined by the phenol–sulfuric acid method.

Molecular structural characteristics measured by SEC-MALLS-RI system

The molecular weight and chain conformation of the EPS in aqueous solution were estimated by SEC columns coupled with multi-angle static laser light-scattering detection (MALLS; λ = 690 nm, Wyatt Technology, Santa Barbara, CA) and a refractive index (RI) detector (Optilab rEX, Wyatt Technology, Santa Barbara, CA). The mobile phase consisted of 0.1 M PBS buffer (pH 6.8) containing 0.04% diaminotetraacetic acid–disodium salt (Na_2EDTA) and 0.01% sodium azide, which was filtered through 0.25 μm filter membranes (Millex HV type, Millipore Co., Bedford, MA). The EPS aqueous solution (1 mg/mL) was injected into two SEC columns (Shodex OH Pack SB-803 and 805 HQ, JM Science Inc., Buffalo, NY) connected in series, and the flowing rate was 5 mL/min. The value of dn/dc (specific refractive index increment) was set as 0.14 mL/g, according to the data in the literature [14]. The data obtained by SEC-MALLS-RI were analyzed by ASTRA 6.1 software (Wyatt Technology, CA). Chain conformation of the polysaccharides in aqueous solution can be identified by calculating the slope between molar mass (M_w) and molecular radius. When the slope of this plot is close to 0.33, the polysaccharides in solution has a spherical conformation. When the slope of the line is 0.5–0.6, the polysaccharides have a random coil conformation insolvent, and when the slope of the line larger than 0.6, the polysaccharides adopt rigid rod-like conformation [12, 14].

Monosaccharide composition analysis

Purified EPS fraction (5 mg) was hydrolyzed with 2 mL of 2 M trifluoroacetic acid (TFA) at 110 °C for 2 h in a sealed glass tube. After reduced with sodium borohydride, acetylated with acetic anhydride and pyridine at 100 °C for 20 min, the resulting alditol acetates were examined by GC-MS (Varian Co., Model Star 3600 CX, Lexington, MA, USA) fitted with a fused silica capillary column (Na form, 300 mm $\times$ 0.25 mm, Supelco Inc., Bellefonte, PA, USA) and a flame ionization detector. The internal standard sugars were also prepared and subjected to GC-MS analysis separately in the same way.

Fig. 1 Effect of three different carbon sources on biomass **a** and EPS production **b** by *S. areolatum* Ehrenb in a 5-L stirred-tank. Identical letters indicate no significant differences ($P < 0.05$)

FT-IR spectroscopy

The Fourier transform infrared (FT-IR) spectroscopy of EPS was performed in the 550 to 4000 cm^{-1} region on a Bruker Tensor 27 FT-IR Spectroscopy. EPS sample was prepared by using the KBr disk method and subjected to 64 scans at 4 cm^{-1} resolution in reference to air to obtain an average spectrum.

Cell proliferation assay

Two kinds of tumor cell lines, human hepatoma Hep G2 cells and human lung carcinoma A549 cells, were cultured in DMEM medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), penicillin (100 U/mL), and streptomycin (100 mg/L) in a humidified atmosphere of 95% air and 5% CO_2 at 37 °C. The proliferation of Hep G2 and A549 were determined using MTT assay, which was the same as previous work [15]. All in vitro results were expressed as the inhibition ratio of tumor cell proliferation as follows:

Antiproliferative activity (%)

$$= \left[1 - \frac{(A_{\text{sample}} - A_{\text{blank}})}{(A_{\text{control}} - A_{\text{blank}})} \right] \times 100\%$$

Table 1 Carbohydrate composition in exopolysaccharides produced during submerged cultivation of *S. areolatum* Ehrenb in the media with selected three different carbon sources

Carbohydrate (%)	Carbon sources in the medium		
	Glucose	Fructose	Sucrose
Rhamnose	nd	0.86 ± 0.69a	0.33 ± 0.05a
Ribose	10.4 ± 0.71a	5.53 ± 0.36c	4.01 ± 0.49b
Xylose	5.66 ± 0.21a	9.81 ± 0.77b	5.84 ± 0.54a
Gluconic acid	40.13 ± 2.79a	47.35 ± 3.05b	47.46 ± 2.94b
Galactose	6.81 ± 0.51a	2.21 ± 0.28c	4.72 ± 0.33b
Glucose	4.60 ± 0.48a	4.21 ± 0.50a	4.53 ± 0.68a
Mannose	30.09 ± 3.21a	29.70 ± 3.79a	31.96 ± 2.15a
Galactose acid	2.31 ± 0.05a	0.51 ± 0.27c	1.15 ± 0.19b

nd not detected

Identical letters indicate no significant differences ($P < 0.05$)

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

Statistics

Data were reported as mean ± S.D. ($n = 3$). Statistical significance was determined by Student's t test. Calculations were carried out by SPSS version 16.0 (version 11.0, Chicago, IL). Analysis of variance (ANOVA) was applied for determining differences between the results of samples. Values of $p < 0.05$ were considered as significantly different.

Results

Effect of carbon sources on biomass and EPS production

Figure 1 shows the biomass and EPS by *S. areolatum* Ehrenb on three different carbon sources in a 5-L stirred-tank. After a fermentation period of 12 days, sucrose was the most favorable carbon source for biomass production. However, the highest EPS yield of 1.35 g/L achieved when fructose was employed. The result indicates that *S. areolatum* Ehrenb has the ability to consume various carbon sources, all of which have different stimulatory effect on biomass and EPS production.

Effect of carbon sources on EPS molecular properties

The EPS was obtained from submerged culture of *S. areolatum* Ehrenb was purified by gel filtration chromatography on Sepharose CL-6B; the main fraction of each EPS produced from three different carbon sources was collected. The monosaccharide compositions were determined using GC-MS analysis as illustrated in Table 1. As expected, the monosaccharide composition identified in the EPS varies with the different carbon sources through the predominant monosaccharides were gluconic acid and mannose. The IR spectra for the three EPS have been compiled in Fig. 2. All samples

Fig. 2 The FT-IR spectra of the EPS fractions produced by submerged cultivation of *S. areolatum* Ehrenb in the media with selected three different carbon sources

exhibited similar functional peaks, which are typical to fungal EPS groups.

The relevant molecular parameters of each EPS fraction are summarized in Table 2. In Table 2, the molecular weights of EPSs from glucose, fructose, and sucrose as carbon source in the medium were estimated to be 5.629×10^5 [5], 1.332×10^5 , and 2.732×10^5 [5], respectively. The root mean square (RMS) radius is a measure of the molecule's size weighted by the mass distribution about its center of mass. For each of these moments of the distribution, the RMS radii of the EPSs were calculated. Compared with the peaks of glucose and sucrose,

the RMS radius for the peak of fructose ranged from 21.8–26.2 nm was smaller (Table 2).

Effect of carbon sources on antiproliferative activities

Many studies revealed that polysaccharides from medicinal fungi have shown direct antiproliferative effect on tumor cells [16, 17]. Antiproliferative activities of *S. areolatum* Ehrenb EPS are shown in Fig. 3. It was observed that antiproliferative activities of *S. areolatum* Ehrenb increased significantly for both treating Hep G2 cancer cells (Fig. 3a) and human lung carcinoma A549 cells (Fig. 3b) in a dose-dependent matter. The antiproliferative activities of EPS with fructose as carbon source were superior to those observed for other two EPSs irrespective of the concentration of the fraction ($p < 0.05$), reaching a maximum of 41.9% for treating Hep G2 cancer cells and 23.6% for treating human lung carcinoma A549 cells at 500 $\mu\text{g/mL}$.

Discussion

It has been reported that different carbon sources could have different pathway and have different influences of catabolic repression on the secondary metabolism [18, 19]. Unlike sucrose, both glucose and fructose are hexoses and can participate in glycolytic and pentose phosphate pathways. According to biomass and EPS yield in Fig. 1, sucrose was shown to sustain high mycelia growth rates due to high carbon conversion efficiency during fermentation. Though it is well

Table 2 Relevant molecular parameters of exopolysaccharides produced by submerged cultivation of *S. areolatum* Ehrenb in the media with selected carbon sources in MALLS analysis

Parameters ^a	Carbon sources in the medium		
	Glucose	Fructose	Sucrose
M_n^a (g/mol)	1.240×10^5	2.776×10^4	1.473×10^4
M_w^a (g/mol)	5.629×10^5	1.332×10^5	2.732×10^5
M_z^a (g/mol)	8.806×10^6	1.357×10^6	3.474×10^7
M_w/M_n^b	4.538	4.797	18.552
R_n^c (nm)	37.8	26.2	35.6
R_w^c (nm)	34.4	22.0	33.3
R_z^c (nm)	55.0	21.8	49.5

^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. 3 The double logarithmic plots of root mean square radius vs molecular mass for exopolysaccharides of produced by submerged cultivation of *S. areolatum* Ehrenb in the media with selected three different carbon sources in MALLS analysis

known that sucrose is a disaccharide combination of the monosaccharides glucose and fructose, the knowledge concerning the metabolic utilization of sucrose for the production of biomass and EPS is limited. In this work, it was observed that different carbon sources do affect the yield or structure of EPS. As studied by Chen et al. [20], who found different carbon source may influence the EPS yield of *Cordyceps sinensis* Cs-HK1. Their results indicated that the addition of galactose improved the total yield of EPS in fermentation culture while mannose could increase the content of glucose in EPS.

As shown in Table 1, the highest monosaccharide contents of xylose and ribose were observed in monosaccharide composition when the fructose and glucose were employed, respectively, as carbon source. The results indicate that EPS composition is slightly affected by carbon sources. Kai et al. found that carbon sources can influence heteropolysaccharides synthesis by *Pestalotiopsis microspora* [18]. When glucose was used as the carbon source, polysaccharides containing large amounts of mannose units were formed. While with xylose as the carbon source, a considerable amount of mannose and galactose units were produced. Wu et al. also reported similar results when different carbon source were used to produce polysaccharide by *Cordyceps sinensis* fungus Cs-HK1. The glucose contents of EPS were 76% with the addition of mannose, 59% with galactose, compared with 62% with glucose only [20]. Sutherland stated that some enzymes should be involved in EPS biosynthesis [21]. Mozzi et al. reported that EPS synthesis by *Lactobacillus casei* CRL 87 was correlated with the activity of UDP-glucose pyrophosphorylase and UDP-galactose 4-epimerase [10].

As showed in Fig. 2, all EPSs displayed a broad-stretching characteristic peak at ca. $3299\text{--}3305\text{ cm}^{-1}$ for -OH groups and a weak C-H stretching band at ca. $2929\text{--}2930\text{ cm}^{-1}$. Further, an asymmetrical stretching peak was noticed at ca. $1645\text{--}1651\text{ cm}^{-1}$ suggesting the presence of the carbonyl group (C=O). All these absorption peaks are common features of

polysaccharides. Specifically, the peaks at $810\text{--}811\text{ cm}^{-1}$ and 917 cm^{-1} for EPS were assigned to mannose and α -D-glucans, respectively [22]. The FT-IR absorption spectra of all EPSs are slightly different, indicating that their chemical structures are similar. According to the results of molecular weight (Table 2), it indicated that carbon source has an influence on the molecular weights of EPS instead of their conformation. Vaningelgem et al. suggested that molecular weight of EPS is very important for studying their structure-function relationship [23]. Zhang et al. reported that the EPS with high molecular weight could not exert bioactivity by limiting its cell membrane permeability [24].

Polysaccharides are polydispersed molecules due to their wider molecular weight ranges. The molar mass dispersity (M_w/M_n), a measure of the width of molecular mass distribution, is a ratio of weight average molecular weight divided by the number average molecular weight. The polydispersity ratio (4.797) of EPS with fructose was similar to that of EPS with glucose and much lower than that of EPS with sucrose, which means that the molecule of EPS with fructose and glucose exist much less dispersed in aqueous solution and does not form large aggregates. This information is important because many bioactivities of polysaccharides can be greatly influenced by the molecular weight distribution. The slope for EPS in the double logarithmic plots of RMS radius versus molecular mass was shown in Fig. 3. The values of slope of EPS indicated 0.13–0.28, which implies that the EPS molecule exists as a nearly globular shape in aqueous solution (see SEC/MALLS ANALYSIS).

It has been reported that the biological activities of polysaccharides derived from fungi were closely related to solubility in water, molecular size, branching frequency, and forms [25, 26]. The superior antiproliferative activities of EPS fraction with fructose as carbon source as compared with the other two EPS fractions (showed in Fig. 4) may be attributed to the differing molecular properties. The larger content of xylose in EPS fraction with fructose as carbon source could be of

Fig. 4 Antiproliferative activity of EPS fractions produced by submerged cultivation of *S. areolatum* Ehrenb in the media with selected three different carbon sources. The results represent mean $\pm$ S.D. ($n = 3$). Hep G2 cancer cells **a** and human lung carcinoma A549 cells **b**

relevance to account for the essentially different antiproliferative properties. Similarly, Chen et al. investigated the EPS of *Tremella mesenterica* from submerged cultures with various carbon sources and found that xylose and glucose were better carbon sources from the viewpoint of immunomodulatory activity [27]. Deng et al. reported the antiproliferative activity of *Lycium barbarum* polysaccharide was closely related to its molecular weight, and polysaccharide with moderate MW (4×10^4 [4] – 3.5×10^5 [5]) was the main active fraction [28]. As a whole, for the EPS isolated from *S. areolatum* Ehrenb, compositional xylose and relatively low molecular mass would be helpful to the enhancement of antiproliferative activity. The results suggest that carbon source can be applied to vary both the molecular properties and the bioactivity of EPS.

Conclusion

The application of three different carbon sources may influence the yield, composition, molecular weight, and antiproliferative activity of EPS from the submerged culture of *S. areolatum* Ehrenb. The results suggested that the molecular properties and antiproliferative activities vary with the different carbon sources. Fructose is better carbon source from the viewpoint of antiproliferative activity due to the relatively high content of xylose in EPS with low molecular weight. Thus, the alternate carbon-source strategy may be successfully used for the regulation of biosynthesis and activity by *S. areolatum* Ehrenb. Further works in our laboratory should be conducted to investigate the EPS biosynthetic pathways and structure-function relationship of EPS from the different carbon source.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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