Process Biochemistry 48 (2013) 380–386

Contents lists available at SciVerse ScienceDirect

Process Biochemistry

journal homepage: www.elsevier.com/locate/procbio

Fractionation, characterization and antioxidant activity of exopolysaccharides from fermentation broth of a *Cordyceps sinensis* fungus

Qi-Lin Huang[a,b], Ka-Chai Siu[a], Wen-Qiang Wang[a], Yi-Ching Cheung[a], Jian-Yong Wu[a,*]

[a] Department of Applied Biology & Chemical Technology, The Hong Kong Polytechnic University, Hung Hom, Kowloon, Hong Kong
[b] College of Food Sciences and Technology, Huazhong Agricultural University, Wuhan 430070, China

ARTICLE INFO

Article history:
Received 27 September 2012
Received in revised form 3 December 2012
Accepted 2 January 2013
Available online 10 January 2013

Keywords:
Cordyceps sinensis
Liquid fermentation
Ethanol precipitation
Polysaccharide–protein complex
Antioxidant activity

ABSTRACT

Exopolysaccharide (EPS) fractions $P_{1/5}$, $P_{2/5}$, P_1, P_2 and P_5 were isolated from the fermentation medium of a medicinal fungus *Cordyceps sinensis* by gradient precipitation with ethanol at 1/5, 2/5, 1, 2, and 5 volume ratios to the liquid medium. $P_{1/5}$ and $P_{2/5}$ were mainly composed of polysaccharides with negligible protein content and a large molecular or particle size (intrinsic viscosity [η] 2025 mL/g; hydrodynamic radius R_h 905 nm). The fraction attained at a higher ethanol volume ratio had a much higher protein and lower carbohydrate content, and a smaller molecular size. In particular, P_5 was composed of mainly protein with an average molecular weight 16 kDa, [η] 4.3 mL/g and R_h 23.5 nm. The antioxidant activities of EPS fractions showed a significant dependence on the protein content, being negligible of $P_{1/5}$ and $P_{2/5}$, low to moderate of P_1 and P_2, and very strong of P_5. Gradient ethanol precipitation was proven a simple and workable method for initial fractionation of polysaccharides, proteins and their complexes with different molecular sizes and for further identification of the bioactive components.

1. Introduction

Polysaccharides (PS) and their protein complexes (PSPs) derived from edible and medicinal fungi have a wide range of nutraceutical functions and medicinal properties including antitumor, immunomodulatory and antioxidant activities [1–3]. The fungal PS are usually extracted from two major source materials, mushrooms or fungal fruit bodies grown on plant substrate and fungal mycelium produced by fermentation. Liquid or submerged fermentation of fungal mycelia has been widely explored for mass production of fungal biomass and useful metabolites such as exopolysaccharides (EPS) from some of the most well-known and important medicinal fungi, *Trametes* (*Coriolus*) *versicolor* [4], *Ganoderma lucidum* [5] and *Cordyceps* spp. [6,7].

Although many studies have demonstrated the bioactivities of fungal PS, information is still limited about the mechanisms of action and the structure-activity relationships. Inconsistent or controversial results have been reported from different studies on the activity level and the correlation to the molecular properties of PS extracted from the same species [8,9]. A major obstacle for the quantification and understanding of their activities is the complex and unclear chemical composition of the PS fractions applied in previous studies, which were mostly crude mixtures of PS, proteins and PSPs in wide molecular weight (MW) ranges. In this regard, it is essential to develop and use effective methods for fractionation and purification of PS. Organic solvent precipitation is a common method for isolation and fractionation of biopolymers such as PS, proteins and PSPs from aqueous solutions [10–12]. Among various organic solvents, ethanol is the most favorable in laboratory and industry due to its relatively low cost, low toxicity to human and desirable physicochemical properties [12,13]. The volume of ethanol required for the precipitation of a given PS depends mainly on its MW and the precipitation conditions and, in most cases, 3–5 volume ratios of ethanol to PS solution (70–80% v/v) for complete precipitation of PS in the mushroom extract solutions and EPS in the liquid fermentation media [14].

Cordyceps sinensis (Berk.) Sacc., generally known as the Chinese caterpillar fungus, is a rare and special mushroom with a fruiting body formed on an insect larva which is mainly distributed on the Tsinghai-Tibet high plateaus of western China [15]. It is a precious medicinal fungus in Chinese medicine and used as a tonic for a broad spectrum of health benefits and pharmacological functions [16]. Because of the high medicinal value and the limited resource of natural *C. sinensis* organisms, mycelial fermentation has become a major source of *C. sinensis* fungus and components.

Abbreviations: ABTS, 2,2′-Azinobis (3-ethylbenzothiazoline-6-sulfonic acid); DOSY-NMR, Diffusion ordered spectroscopy-NMR; DLS, Dynamic light scattering; EPS, Exopolysaccharide; FRAP, Ferric reducing ability of plasma; GPC, Gel permeation chromatography; R_h, Hydrodynamic radius; [η], Intrinsic viscosity; MW, Molecular weight; PAS, Periodic acid-Schiff; PS, Polysaccharide; PSPs, Polysaccharide–protein complexes; TEAC, Trolox equivalent antioxidant capacity.

* Corresponding author. Tel.: +852 3400 8671; fax: +852 2364 9932.
E-mail address: bcjywu@polyu.edu.hk (J.-Y. Wu).

1359-5113/$ – see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.procbio.2013.01.001

Cs-HK1 is a *C. sinensis* species isolated from a natural fruiting body and Cs-HK1 mycelial culture has been established in our lab. The Cs-HK1 mycelial culture applied to liquid fermentation produced up to 4–5 g/L EPS, which exhibited significant antioxidant and immunomodulatory activities [17,18]. However, the EPS was isolated from the Cs-HK1 culture medium by a single-step precipitation with 4–5 volumes of ethanol, yielding a crude mixture of PS, proteins and PSPs in a wide MW range. It is not clear how the proteins were associated with the PS and how the different MW fractions and chemical constituents of the EPS contribute to the bioactivities.

In this study, gradient precipitation with increasing volume of ethanol was applied to isolate and fractionate the EPS from the Cs-HK1 fermentation medium into different MW fractions. The chemical composition and essential molecular properties including molecular weight, intrinsic viscosity and hydrodynamic radius in an aqueous solution were analyzed and their antioxidant activities were determined with chemical and cell culture assays. Moreover, diffusion ordered spectroscopy (DOSY)-NMR technique was employed, in addition to gel permeation chromatography (GPC), to attain more full MW spectra of the EPS fractions and to monitor the process of gradient precipitation.

2. Materials and methods

2.1. Fungal species and submerged fermentation

The Cs-HK1 fungus was previously isolated from the fruiting body of a natural *Cordyceps sinensis* and its stock culture was maintained on solid potato–dextrose–agar medium at 4 °C [19]. The seed culture of Cs-HK1 for liquid fermentation was inoculated with the mycelial spores taken from the solid stock culture using an inoculation loop into a liquid medium contained in Erlenmeyer flasks, and incubated on a rotary shaker at 150 rpm and 20 °C for 7 days. The liquid medium was composed of 40 g/L sucrose, 10 g/L yeast extract, 5 g/L peptone and a few inorganic salts with an initial pH 6.8 as reported previously [19]. The mycelial culture broth was then transferred into a 15 L stirred and aerated fermenter (Biostat®C, Satorius, Germany) filled with 8 L of liquid medium, which was operated for 6 days at 20 °C, an air flow rate of 1 vvm and dissolved oxygen (DO) above 20% air saturation. At the end of fermentation, the mycelial broth from the fermenter was spun down at 8000 rpm for 20 min and the supernatant liquid medium was collected. The mycelial fermentation was run twice under the same conditions, yielding a total of 10 L supernatant liquid medium for the isolation of EPS.

2.2. Isolation of EPS fractions by gradient ethanol precipitation

The liquid fermentation medium (10 L) was filled into a 20 L barrel placed on a magnetic stirrer. Ethanol (95% grade) was added slowly at 1/5 volume ratio to the liquid medium, and mixed with constant stirring at room temperature (20–25 °C) for 3 h, during which the solution gradually turned cloudy. The solution was then kept stationary at 4 °C for overnight, followed by centrifugation at 10,000 rpm for 20 min. The precipitate was collected and the supernatant was subjected to the next step of precipitation with a higher ethanol volume ratio. In this way, the precipitated fractions were collected successively at 1/5, 2/5, 1, 2, and 5 ethanol-to-liquid medium volume ratios, designated $P_{1/5}$, $P_{2/5}$, P_1, P_2, and P_5, respectively. The final supernatant retained at 5 volume ethanol after the removal of P_5 was also collected, concentrated and freeze-dried, designated fraction S. All EPS precipitates and S were freeze-dried and stored at 20–25 °C for further analysis and tests.

2.3. Analysis of chemical constituents

The total sugar content of EPS fractions was determined by the Anthrone test using glucose as a reference and the protein content determined by the Lowry method using bovine serum albumin as a reference, as reported previously [17]. The protein constituents of EPS fractions were detected by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), using a 4.5% stacking gel and 12.5% separation gel. The protein markers for the SDS-PAGE contained 10 pre-stained recombinant proteins in the MW range of 10–170 kDa (Fermentas Life Science #SM0671). The EPS sample was dissolved at 10 mg/mL in distilled water and added at 1:3 volume ratio into a buffer solution of 0.5% SDS with 1%-mercaptoethanol, and then heated to boiling for 5 min. The gels were stained with Coomassie Brilliant Blue R-250 to visualize proteins and with periodic acid-Schiff (PAS) stain to visualize PSPs.

2.4. Determination of molecular parameters

2.4.1. Intrinsic viscosity ([η]) by viscometry

Intrinsic viscosity ([η]) was determined by Huggins and Kraemer equations,

$$\frac{\eta_{sp}}{c} = [\eta] + k'[\eta]^2 c \quad (1)$$

$$\frac{\ln \eta_r}{c} = [\eta] - (1/2 - k')[\eta]^2 c \quad (2)$$

where η_{sp}/c is reduced specific viscosity, $(\ln \eta_r)/c$ inherent viscosity, and k' is a constant depending on the molecular properties, solvent and experimental conditions. The viscosity of EPS solution in water was measured at 30 °C with an Ubbelohde capillary viscometer. As the effluent time for solvent was over 120 s, the kinetic energy correction was negligible.

2.4.2. MW by GPC analysis

High-pressure gel permeation chromatography (HPGPC) was performed of the EPS fractions on a Waters instrument consisting of a Waters 1515 isocratic pump and a Waters 2414 refractive index detector with two ultrahydrogel columns 250 and 2000 (7.8 × 300 mm, Waters Corp., Milford, MA) in series at 30 °C. Water (filtered through 0.22 μm membrane and degassed) was used as the mobile phase at a flow rate of 0.6 mL/min. All sample fractions were completely dissolved in water (2 mg/mL for $P_{1/5}$ and $P_{2/5}$, 5 mg/mL for P_1, P_2, P_5 and S) and filtered through 0.45 μm membrane before the injection (injection volume 20 μL). The MW values of EPS fractions were derived from the calibration curve measured with linear Dextran MW standards 1, 5, 12, 50, 150, 670 and 1500 kDa (Sigma).

2.4.3. Hydrodynamic radius (R_h) by DLS

Dynamic light scattering (DLS) measurement was performed of the EPS fractions at 25 °C on a Malvern Zetasizer Nano (Malvern Instruments Ltd., UK) at 632.8 nm and 90° scattering angle. The sample was dissolved in water at 0.5 mg/mL. The analysis of intensity autocorrelation function was performed with a Laplace inversion program (CONTIN). A total of five runs were performed for each measurement and the data were processed with the MASOPTION software. The average hydrodynamic radius R_h of polymer particles was derived from the Stokes–Einstein equation [20].

2.4.4. DOSY-NMR experiments

DOSY (Diffusion Ordered Spectroscopy)-NMR analysis was performed to gain gross information of the molecular sizes of the EPS fractions. The DOSY method measures the translational self-diffusion of molecules in solution with which the NMR spectra of the components of a mixture can be separated according to their apparent diffusion coefficients (D) [21]. The diffusion coefficient is correlated to the hydrodynamic radius by the Stokes–Einstein equation $D = k_B T/(6\pi\eta_s R_h)$. The method has been employed to monitor the fractionation process of PS from crude mushroom extracts [22].

The EPS fractions $P_{1/5}$ and $P_{2/5}$ were dissolved in 99.9% D_2O at 2 mg/mL, and all other EPS fractions were dissolved in D_2O at 4 mg/mL. The NMR analysis was performed at 25 °C on a Bruker AVANCE AV600 NMR spectrometer, equipped with a Bruker triple resonance z-gradient inverse probe head. The DOSY experiment was performed by recording 8 scans for each gradient step with the ledbpg2s pulse sequence, a linear gradient of 32 steps between 2% and 95%, a diffusion time (big delta) of 0.1–0.4 s and the length of the square diffusion encoding gradient pulses (little delta) of 1–2 ms, and a total acquisition time of ~20 min. The standard Bruker protocol was used for data processing with Topspin 2.1. The sum of the projections in the diffusion dimension was displayed by using the standard f1sum command over the whole 2D-DOSY spectra. In these projections, the x-axis shows the values of log D instead of retention time values as for the classical chromatography.

2.5. Antioxidant activity assays

Three methods were employed to measure the antioxidant activities of various fractions, including the Trolox equivalent antioxidant capacity (TEAC) assay, the ferric reducing ability of plasma (FRAP) assay and cytoprotection test in cell culture. All reagents used for the antioxidant assays were of analytical grade and purchased from Calbiochem/EMD (Gibbstown, NJ, USA) unless otherwise stated. All aqueous media and reagent solutions were prepared with distilled water.

TEAC assay measures the ability of a sample to scavenge $ABTS^{\bullet+}$ radicals with Trolox [(S)-(2)-6-hydroxy-2,5,7,8-tetramethyl-chroman-2-carboxylic acid] as an antioxidant reference, and the activity was expressed in μmol Trolox/g sample. FRAP assay measures the reducing power of a sample by transferring a single electron to $Fe(TPTZ)_2(III)$, resulting in $Fe(TPTZ)_2(II)$, and the activity was expressed in μmol $FeSO_4$/g sample. Both assays were performed with the EPS fractions predissolved in water at several different concentrations from 25 to 2500 mg/L. The procedures and conditions for the TEAC and FRAP assays were similar to those reported by Leung et al. [17]. In brief, the TEAC activity of the fractions was tested against $ABTS^{\bullet+}$ radicals, which were generated from the oxidation of 2,2′-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) by potassium persulphate ($K_2S_2O_8$). The EPS sample solution (980 μL) was mixed with the diluted $ABTS^{\bullet+}$ solution (20 μL) for 2 h, followed by measurement of the absorbance at 734 nm. The absorbance value was converted to TEAC value in μmol Trolox/g sample by the