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Structure identification of α-glucans from *Dictyophora echinovolvata* by methylation and 1D/2D NMR spectroscopy

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ABSTRACT

Dictyophora echinovolvata is a kind of edible mushroom in the *Dictyophora* genus, of which polysaccharide is an important chemical substance. Herein, three polysaccharide fractions (DEP-4P, DEP-6P and DEP-8P) were prepared from water extract of *D. echinovolvata* using gradient ethanol precipitation method. Their chemical structures were analyzed by methylation analysis and 1D/2D NMR spectroscopy. Molecular weights were determined by multi-angle laser light scattering combined with size-exclusion chromatography (HPSEC-MALLS). HPSEC data showed that DEP-4P had the highest values of molecular weight, intrinsic viscosity and hydrodynamic radius. DEP-6P and DEP-8P had lower molecular weights, which contributed to their easily distinguished 1D and 2D NMR spectra. Methylation and NMR analysis suggested that the three fractions were linear α-(1 → 4)-glucans with α-Glc*p* residues linked to the backbone at *C*-6. Differences among the three fractions were the molar ratios of the identified glycosidic bonds. Repeating units of polysaccharides from *D. echinovolvata* were proposed as follows:

$$\Big[\rightarrow 4)\text{-}\alpha\text{-Glc}p(\text{-}1\Big[\rightarrow 4)\text{-}\alpha\text{-Glc}p(\text{-}1\Big]_m\rightarrow 4)\text{-}\alpha\text{-}\underset{\substack{6\\ \uparrow \\ \alpha\text{-Glc}p}}{\text{Glc}p}(\text{-}1\rightarrow 4)\text{-}\alpha\text{-Glc}p(\text{-}1\Big]_n\rightarrow$$

1. Introduction

Consumption of mushrooms has become a fashion all around the world for a long time (Kalač, 2009; Meng, Liang, & Luo, 2016; Ruthes, Smiderle, & Iacomini, 2016). Edible mushrooms with distinctive shapes are popular not only because of their pleasant taste, but also the high nutritive values. Bioactive polysaccharides with various types of chemical structures were successfully isolated and identified from edible mushrooms, such as those from *Basidiomycetes* class and *Ascomycetes* class (Ferreira et al., 2015; Kalač, 2009; Meng et al., 2016; Ruthes et al., 2016; Singdevsachan et al., 2016; Zhang, Cui, Cheung, & Wang, 2007). Among the edible mushrooms, *Dictyophora indusiata* is one of the most famous species in *Dictyophora* genus. Current studies show that *D. indusiata* has many bioactivities, including immunoregulation, antioxidant activity, antityrosinase, anti-inflammation and neuroprotective activity, which benefit from many natural bioactive substances (furfural, polysaccharides, dictyophorines, et al.) (Deng et al., 2012; Hara, Kiho, Tanaka, & Ukai, 1982; Kawagishi et al., 1997; Sharma, Choi, Sharma, Choi, & Seo, 2004). Particularly, polysaccharides from *D. indusiata* exhibited promising functional properties, which were attributed to the special chemical structures (Ker, Chen, Peng, Hsieh, & Peng, 2011).

Since 1980s, polysaccharides from *D. indusiata* have been identified to be partially *O*-acetylated α-(1 → 3)-mannans, (1 → 6)-branched β-(1 → 3)-glucans and fucomannogalactan (Hara & Ukai, 1995; Hara et al., 1982; Ukai, Hara, & Kiho, 1982; Ukai, Hara, Kiho, & Hirose, 1980). Later, a β-(1 → 3)-glucan with (1 → 6)-β-glucopyranosyl side chains existing as a triple helical chain in water was isolated from the fruiting body of *D. indusiata* (Wang, Xu, Zheng, & Chen, 2009). Recently, polysaccharides with other structural characteristics, including DIPs-2 with a backbone of β-(1 → 6)-mannopyranosyl residues and DIP with β-(1 → 6)-linked glucoses as the main linkages from *D. indusiata* were reported (Deng et al., 2012; Hua et al., 2012; Ker et al., 2011; Liao et al., 2015; Wang, Zhang, Sun, & Zhang, 2015).

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D. echinovolvata is another important member belonging to the *Dictyophora* genus. It has smaller fruit body than *D. indusiata*, and the two species have significant differences in the size and shape of indusium (Fig. 1s in Supplementary materials). Unlike *D. indusiata*, which has been widely studied, there are only a few studies focusing on the chemical structures of polysaccharides from *D. echinovolvata*. In 2001, a purified polysaccharide (DE2-2) from the fruit bodies of *D. echinovolvata* was determined to contain glucose, mannose, galactose and fucose in a molar ratio of 8.68:1.00:1.85:0.74 with molecular weight of 84,000 Da (Lin, Yu, & Liu, 2001). Later in 2013, the polysaccharide from pileus of *D. echinovolvata* was reported to have high antioxidant activity by Fan et al. (2013). Till now, the fine structural characteristics of polysaccharides from *D. echinovolvata* are still unknown. To extend the understanding of *D. echinovolvata* and compare the functional properties of carbohydrate polymers from mushrooms in *Dictyophora* genus, much work in the structural and bioactive aspects need to be done.

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.foodchem.2018.07.160.

In our previous work, three purified fractions (DEP-4P, DEP-6P, DEP-8P) were isolated from the crude water-soluble extract of *D. echinovolvata* using gradient ethanol precipitation method. They were deduced to be α-glucans with different molecular weights and morphological properties (Zhang, Li, & Shi, 2017). The current study aims to elucidate the detailed structural characteristics of the three fractions by means of methylation and NMR analysis and then detect the accurate molecular weights and viscosities using high performance size exclusion chromatography (HPSEC) coupled with multiple detectors: a multi-angle laser light scattering detector (MALLS), a viscometer (Vis) and a refractive index detector (RID).

2. Materials and methods

2.1. Materials and reagents

D. echinovolvata was bought from local market in Gutian county, Fujian province, China. Crude water-extract of *D. echinovolvata* was prepared in our lab using hot water-extraction and ethanol precipitation methods. DEP-4P, DEP-6P and DEP-8P were prepared using gradient ethanol precipitation as described in the previous report (Zhang et al., accepted). Briefly, the extract was redissolved in distilled water, and ethanol was added slowly to the solution to a concentration of 20% (v/v). After 12 h, the first precipitate after centrifugation was discarded and ethanol was added to the supernatant to a concentration of 40% (v/v). Therefore, we obtained the second precipitate, which was washed by absolute ethanol, and then redissolved, dialyzed and freeze-dried to generate the first fraction named as DEP-4P. DEP-6P and DEP-8P were collected in the same manner.

Methyl iodide (catalog number, 456756), deuterium oxide (99.9% D, catalog number, 151882) and sodium borodeuteride (98 atom% D, catalog number, 205591) were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). Trifluoroacetic acid (TFA, catalog number: T103291) of analytical grade was bought from the Aladdin Industrial Corporation (Shanghai, China). Aqueous solutions were prepared using ultrapure water from a Milli-Q water purification system (Millipore, Bedford, MA, USA). All other reagents were of analytical grade unless otherwise specified.

2.2. Methylation and GC–MS analysis

Methylation of polysaccharides was conducted according to the method derived from Ciucanu and Kerek (1984) with some modifications (Kang et al., 2011; Xing et al., 2014; Zhang et al., 2017). Briefly, 2–3 mg vacuum-dried sample was fully dissolved in 2.0 mL DMSO, to which 30 mg of NaOH dry powder was added. After incubation under stirring for 2 h, 0.8 mL of iodomethane was added slowly in the ice bath. The reaction was kept in the dark for 3 h. Then, a drop of deionized water was added to stop the reaction. Chloroform was used to extract the methylated polysaccharide, which was then dried and detected by FT-IR spectrum to make sure that the peak of hydroxyl group at 3200–3700 cm^{-1} disappeared completely. The methylated polysaccharide was hydrolyzed by 4 M TFA at 100 °C for 6 h and then reduced with sodium borodeuteride and acetylated with acetic anhydride. The generated partially methylated alditol acetates (PMAAs) were subjected to a GC–MS apparatus (Thermo Quest Finnigan, San Diego, CA) having an SP-2330 capillary column (Supelco, Bellefonte, PA). The temperature programming condition of GC and the ion trap MS detector was referring to the method reported by Zhang, Nie, Yin, Wang, and Xie (2014).

2.3. NMR spectroscopy

Polysaccharides (30 mg) dissolved in D_2O (99.8%) were lyophilized to exchange hydrogen with deuterium for three times. The samples were finally dissolved in 0.5 mL D_2O (99.9%) and transferred into a 5-mm nuclear tube. The ^1H, ^{13}C and DEPT (distortionless enhancement by polarization transfer) NMR spectra were recorded at 400.13 and 100.61 MHz, respectively, on a Bruker Avance 400 MHz NMR spectrometer (Brucker, Rheinstetten, Germany) at around 295 K. Two dimensional experiments, including the homonuclear 1H/1H correlation spectroscopy (COSY), heteronuclear single-quantum coherence (HSQC), heteronuclear multiple-bond correlation (HMBC) and nuclear Overhauser effect spectroscopy (NOESY) using the standard Bruker pulse sequence were also conducted for further analysis. The 1D NMR spectra were recorded by suppressing the ^1H and ^{13}C signals at 2.15 ppm and 30.2 ppm of acetone, respectively.

2.4. Molecular weights detected by HPSEC-MALLS-Vis-RID

The high performance size-exclusion chromatograph (HPSEC) (Wyatt Technology Co., Santa Barbara, CA, USA) equipped with the same online detectors as previously reported (Yin et al., 2015) was used for molecular weights determination. A Wyatt Model 1500 dual pump (Wyatt Technology Co, Santa Barbara, CA, USA), an Ohpak SB-G guard column (8 mm × 50 mm) and two analytical columns (8 mm × 300 mm): Ohpak SB-806 HQ column and Ohpak SB-804 HQ column (Showa Denko K.K., Tokyo, Japan) were equipped in the HPSEC system. The temperature of columns was kept at 35 °C, and temperature of three detectors was 25 °C. The mobile phase was 0.1 M $NaNO_3$ containing 0.02% (w/w) at a flow rate of 0.6 mL/min. Samples (2.0 mg/mL or 5.0 mg/mL) dissolved in mobile phase were prepared by passing through a 0.22 μm membrane filter before injected into the chromatographic system. Each sample was subjected for three runs and the average results (mean ± SD) were reported. Data was analyzed using ASTRA (Version 6.1.7) software, normalized with the dextran standard (M_w = 35,000–45,000 Da, Lot number, SLBD3835V) from Sigma-Aldrich Co. (St. Louis, MO, USA). A refractive index increment (d*n*/d*c*) of 0.146 mL/g was used in the calculation.

3. Results and discussion

3.1. Methylation analysis

After methylation, de-polymerization and conversion, the obtained PMAAs of DEP-4P, DEP-6P and DEP-8P were analyzed by GC–MS. The total ion chromatogram was provided in the Supplementary material (Fig. 2s). Individual peaks from the gas chromatogram and mass spectrum were identified by the retention time and the mass spectra patterns from literatures (Ishurd et al., 2010; Jiang et al., 2016; Li, Yan, Hua, & Zhang, 2013; Niu, Yan, Lv, Yao, & Yu, 2013; Wang et al., 2017). Results of methylation and GC–MS analysis are summarized in Table 1. Three major methylated sugar residues were found, which were