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Characterization and discrimination of polysaccharides from different species of *Cordyceps* using saccharide mapping based on PACE and HPTLC

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ABSTRACT

Polysaccharides from seven species of natural and cultured *Cordyceps* were firstly investigated and compared using saccharide mapping, partially acidic/enzymatic (α-amylase, β-glucanase and pectinase) digestion followed with polysaccharide analysis by using carbohydrate gel electrophoresis (PACE) and high performance thin layer chromatography (HPTLC) analysis, respectively, to obtain the comprehensive profiles of hydrolysates of the polysaccharides and their characters. The results showed that 1,4-α-D-glucosidic, 1,4-β-D-glucosidic and 1,4-α-D-galactosidic linkages were existed in natural and cultured *Cordyceps sinensis*, cultured *Cordyceps militaris*, natural *Cordyceps gracilis* and *Cordyceps ciecadae*. The similarity of polysaccharides from cultured *C. militaris* to natural *C. sinensis* was relatively high, which might contribute to the rational use of *C. militaris*. Moreover, different species of natural and cultured *Cordyceps* can be differentiated based on the saccharide mapping, which is helpful to well understand the structural characters of polysaccharides from different species of *Cordyceps* and to improve the quality control of polysaccharides in natural and cultured *Cordyceps*.

1. Introduction

Cordyceps, one of the well-known tonic foods and traditional Chinese medicines, is a composite consisting of the stromata of the fungus, *Cordyceps sinensis* (Berk.) Sacc. parasitized on the larva and the dead caterpillar. So far, approximately 90 species of *Cordyceps* have been found in China (Zhong et al., 2009). However, only *C. sinensis* is officially recorded in Chinese Pharmacopoeia (Chinese Pharmacopoeia Commission, 2010). Sure, *Cordyceps* is commonly used in China for prevention and treatment of a variety of diseases (Zhu, Halpern, & Jones, 1998a, 1998b). Indeed, polysaccharides are considered as the main bioactive components of *Cordyceps* (Paterson, 2008; Shashidhar, Giridhar, Udaya Sankar, & Manohar, 2013; Zhong et al., 2009; Zhou, Gong, Su, Lin, & Tang, 2009), which are responsible for lots of bioactivities of *Cordyceps*, such as anti-tumor, immunomodulatory, anti-oxidation, hypoglycemic, and hypolipidemic activities (Shashidhar et al., 2013; Zhong et al., 2009). Up to date, only few works have been performed for comparison of polysaccharides in natural and cultured *C. sinensis* (Guan, Yang, & Li, 2010; Guan, Zhao, Feng, Hu, & Li, 2011) due to the structural complicity of polysaccharides and rare materials. Therefore, comparison and characterization of polysaccharides from various different species of *Cordyceps* are very important for improving the quality control of natural and cultured *Cordyceps*.

Saccharide mapping based on enzymatic hydrolysis followed by high-performance size-exclusion chromatography (HPSEC) analysis has been used for comparison of polysaccharides from natural and cultured *C. sinensis* (Guan et al., 2011). However, HPSEC is difficult to simultaneously separate both polysaccharides and their hydrolysates. In addition, the sensitivity of evaporative light scattering detector (ELSD) and refractive index detection (RID), which are widely used for detection of saccharides without UV absorbance, is poor (Li, Wu, Lv, & Zhao, 2013). Actually, the polysaccharide analysis by using carbohydrate gel electrophoresis (PACE) analysis has been employed for analysis of polysaccharide from *Ganoderma* spp. in our previous studies (Wu, Xie, Hu, Zhao, & Li, 2013), which has been approved as the high sensitive, high resolution and high throughput method for analysis of enzymatic

Abbreviations: ANTS, 8-aminonaphthalene-1,3,6-trisulfonic acid; DP, degree of polymerization; ELSD, evaporative light scattering detector; HPTLC, high-performance thin-layer chromatography; HPSEC, high-performance size-exclusion chromatography; PACE, polysaccharide analysis by using carbohydrate gel electrophoresis; RID, refractive index detection; SMC, simulative mean chromatogram; TFA, trifluoroacetic acid.

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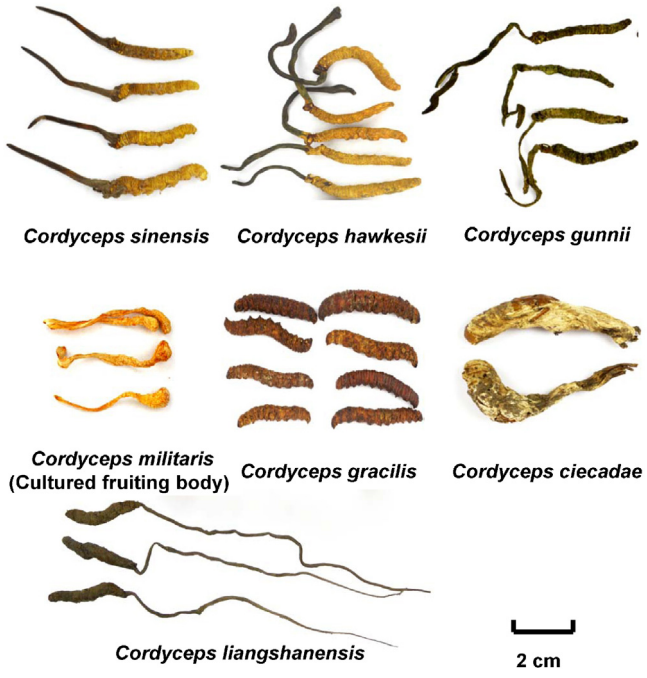

Fig. 1. The typical samples of natural and cultured *Cordyceps*.

hydrolysates containing oligosaccharides. However, the resolution of PACE for analysis of different monosaccharides is poor due to their 8-aminonaphthalene-1,3,6-trisulfonic acid (ANTS) derivatives (Goubet, Jackson, Deery, & Dupree, 2002). Actually, high-performance thin-layer chromatography (HPTLC) is available for separation of different types of monosaccharides (Yang, Guan, Zhang, & Li, 2010), which has been used for analysis of the acid hydrolysates of polysaccharides from two species of *Ganoderma* (Xie et al., 2012). Therefore, the combination of HPTLC and PACE is a good choice for completely understanding comprehensive profile of enzymatic and partial acid hydrolysates of polysaccharides. In this study, polysaccharides from different species of *Cordyceps*, including *C. sinensis*, *Cordyceps militaris*, *Cordyceps gunnii*, *Cordyceps liangshanensis*, *Cordyceps gracilis*, *Cordyceps hawkesii* and *Cordyceps ciecadae* were characterized and compared using saccharide mapping based on HPTLC and PACE analysis, which were helpful to well understand the structural characters of polysaccharides in different species of *Cordyceps* and to improve their quality control of polysaccharides.

2. Materials and methods

2.1. Materials and chemicals

Seven batches (NC1–NC7) of natural *C. sinensis* (NC) and *C. gunnii*, *C. liangshanensis*, *C. gracilis*, *C. hawkesii* and *C. ciecadae* were obtained from different places of China. Nine batches (CM1–CM9) of cultured *C. militaris* (CM) and ten samples (CC1–CC10) of cultured *C. sinensis* (CC) were collected from different manufacturers in China. Other cultured *C. sinensis* samples (CC11 and CC12) were produced in our laboratory (Table 1). Identities of these natural *Cordyceps* spp. were confirmed by Professor Shao-Ping Li, University of Macau, Macau SAR, China. Their characteristic information (Fig. 1) was in accordance with the previous reports (National Institutes for Food and Drug Control & Guangdong Institute for Food and Drug Control, 2011). Species of the cultured *Cordyceps* were certified by State Food and Drug Administration of China or manufacturer. The voucher specimens were deposited at the Institute of Chinese Medical Sciences, University of Macau, Macao, China.

D-Glucose, D-galactose, D-galacturonic acid, soluble starch, cellulose, dextran 2000, dextranase (EC 3.2.1.11), α-amylase (EC 3.2.1.1), β-D-glucanase (EC 3.2.1.6) and cellulase (EC 3.2.1.4) were purchased from Sigma (St. Louis, MO, USA). Laminaribiose (95%), laminaritetraose (95%), laminarihexaose (95%), pectic galactan, oat glucan, pectinase (EC 3.2.1.15) and isoamylase (EC 3.2.1.68) were purchased from Megazyme (Wicklow, Ireland). ANTS was purchased from Tokyo Chemical Industry (Tokyo, Japan). Silica gel 60 TLC plates were obtained from Merk (Merk, Darmstadt, Germany). Polyacrylamide containing a ratio of acrylamide/N,N-methylenebisacrylamide (19:1, w/w) was obtained from Bio-Rad (Hercules, CA, USA). Deionized water was prepared by a Millipore Milli-Q Plus system (Millipore, Bedford, MA, USA). All the other reagents were of analytical grade.

2.2. Preparation of polysaccharides by pressurized liquid extraction (PLE)

The samples were carefully cleaned using a small dry brush, then dried at 40 °C for 24 h, and pulverized via grinding. PLE was performed on a Dionex ASE 200 system (Dionex Corp., Sunnyvale, CA, USA). The powders of sample materials (0.5 g) were mixed with diatomaceous earth in a proportion of 1:1 and placed into an 11 mL of stainless steel extraction cell, then extracted with water under 100 °C for 5 min of static time for one cycle with pressure at 1.034×10^4 kPa. The extract (~20 mL) purged out by nitrogen was transferred into a 25 mL volumetric flask, which was made up to its volume with water. Then the aqueous extract (20 mL) was evaporated to 5 mL of solution under vacuum. Subsequently, ethanol (95%, w/v) was added to the final concentration of 80% (v/v) for precipitation of crude polysaccharides. After standing for 12 h at 4 °C, centrifugation (4500 × *g* for 15 min) was performed. The precipitate was dried on water bath (80 °C), and then redissolved in 5 mL of hot water (80 °C). After centrifugation (4500 × *g* for 15 min), the supernatant was transferred to an ultracentrifugal filter (molecular weight cutoff: 3 kDa, Millipore, Billerica, MA, USA), and then the low molecular weight compounds (Mw < 3 kDa) were removed by centrifugation (4000 × *g*, 20 min, 25 °C). Then, deionized water was added into the supernatant to a total volume of 5 mL. The content of sugar in crude polysaccharides was determined using phenol-sulfuric acid assay with glucose as reference standard (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956). Finally, the crude polysaccharides, which were prepared in duplicates, were obtained for further analysis.

2.3. Partial acid hydrolysis of polysaccharides

The crude polysaccharide solutions (~0.5 mg/200 μL) were treated with trifluoroacetic acid (TFA) at a final concentration of 0.5 mol/L in a total volume of 300 μL. The suspensions were incubated at 80 °C for 5 h. After hydrolysis, the hydrolysates were washed with methanol and evaporated to dryness with a nitrogen evaporator at 35 °C for three times to remove the residue of TFA. The dried products were stored in 4 °C before derivatization with ANTS for PACE analysis, and redissolved in 100 μL of methanol for HPTLC analysis, respectively.

2.4. Enzymatic digestion of polysaccharides

Polysaccharide solutions (~0.5 mg/200 μL) were mixed with certain enzyme (the final concentration of dextranase, β-D-glucanase, isoamylase, α-amylase, cellulase and pectinase were 2 U/mL, 2 U/mL, 20 U/mL, 20 U/mL, 20 U/mL and 20 U/mL, respectively) in a total volume of 300 μL and digested overnight (12 h) at 40 °C. Then the mixtures were heated at 80 °C for 20 min to denature the enzymes. After centrifugation (10,000 × *g*) at room