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Qualitative and quantitative analysis of specific polysaccharides in *Dendrobium huoshanense* by using saccharide mapping and chromatographic methods

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ABSTRACT

Qualitative and quantitative analysis of specific polysaccharides from ten batches of *Dendrobium huoshanense* were performed using high performance size exclusion chromatography coupled with multi-angle laser light scattering and refractive index detector (HPSEC-MALLS-RID), gas chromatography-mass spectrometry (GC–MS), nuclear magnetic resonance (NMR) and saccharide mapping based on polysaccharides analysis by using carbohydrate gel electrophoresis (PACE) and high performance thin layer chromatography (HPTLC). Results showed that molecular weights, the radius of gyrations, and contents of specific polysaccharides in *D. huoshanense* were ranging from 1.16×10^5 to 2.17×10^5 Da, 38.8 to 52.1 nm, and 9.9% to 19.9%, respectively. Furthermore, the main monosaccharide compositions were Man and Glc. Indeed, the main glycosidic linkages were β-1,4-Man*p* and β-1,4-Glc*p*, and substituted with acetyl groups at O-2 and O-3 of 1,4-linked Man*p*. Moreover, results showed that PACE and HPTLC fingerprints of partial acidic and enzymatic hydrolysates of specific polysaccharides were similar, which are helpful to better understand the specific polysaccharides in *D. huoshanense* and beneficial to improve their quality control. These approaches could also be routinely used for quality control of polysaccharides in other medicinal plants.

1. Introduction

Dendrobium huoshanense, a rare and valuable plant of Orchidaceae family, is a specialty of around Huoshan County in Anhui Province of China. The stem of this plant has long been used as traditional medicines for the treatment of salivary, stomach, and ophthalmic disorders [1], and also used as functional food materials to make tea drinks, soups and porridges for the protection of eye and liver [2]. Usually, polysaccharides are considered as the major bioactive compounds in *D. huoshanense*, which possess various bioactivities, such as hepatoprotective [3], immunoregulatory [4] and antioxidant activities [5]. Actually, bioactivities of polysaccharides are closely correlated with their chemical properties, such as molecular weight, types and ratios of compositional monosaccharides, and features of glycosidic linkages [6]. However, starch, a common high molecular weight polysaccharides consisting of a large number of glucose units with α-1,4-glycosidic bonds [7], have also been found in the stems of *D. huoshanense* and its related species [8,9]. Chemical structures of polysaccharides without of removing starch in *D. huoshanense* and its related species have been compared using HPSEC and PACE methods [10,11]. However, qualitative analysis of polysaccharides without of removing starch in *D. huoshanense* may lead to inaccurate results for the ratio of compositional monosaccharides, glycosidic linkages, molecular parameters and saccharide mapping due to the interference of starch. So far, chemical features of specific polysaccharides (non-starch polysaccharides) in *D. huoshanense*, have seldom been systematically investigated and compared. Therefore, qualitative and quantitative analysis of specific polysaccharides in *D. huoshanense* is significantly important, which is helpful for better understanding of chemical characters of bioactive polysaccharides in *D. huoshanense*,

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as well as beneficial to improve their quality control and their performance in both biomedical and functional food fileds.

Chemical characterization such as compositional monosaccharides and glycosidic linkages are usually used for qualitative analysis of polysaccharides relied on variety of analytical methods. For example, saccharide mapping [12] based on polysaccharides analysis by using carbohydrate gel electrophoresis (PACE) and high performance thin layer chromatography (HPTLC) have been developed for the qualitative analysis of polysaccharides from medicinal plants [13] and fungi [14] with high repeatability, stability, sensitivity and throughput. In addition, GC–MS [15] combined with NMR spectroscopy [16] are also feasible and desirable techniques for qualitative and quantitative analysis of compositional monosaccharides and types of glycosidic linkages of polysaccharides. Recently, a rapid and accurate method, high performance size exclusion chromatography coupled with multi-angle laser light scattering and refractive index detector (HPSEC-MALLS-RID) based on refractive index increment (*dn*/*dc*), has been developed for quantitative analysis of polysaccharides based on their response to refractive index detector (RID) and their universal *dn*/*dc* [17,18]. Especially, in this method, individual polysaccharides standard, as well as calibration curve is not required. In this study, qualitative and quantitative analysis of specific polysaccharides from ten batches of *D. huoshanense* was performed using the combination of colorimetric assay with iodine and potassium iodide, GC–MS, NMR, saccharide mapping based on PACE and HPTLC, and HPSEC-MALLS-RID based on *dn*/*dc* analysis.

2. Material and methods

2.1. Materials and chemicals

Ten batches of cultivated *D. huoshanense* (DH01-DH10) were collected in Huoshan, Anhui, China. Identity of the cultivated *D. huoshanense* was confirmed by Professor Naifu Chen, West Anhui University, Anhui, China. The voucher specimens were deposited at the Institute of Chinese Medical Sciences, University of Macau, Macau SAR, China.

D-Glucose (Glc), D-rhamnose (Rha), D-xylose (Xyl), D-arabinose (Ara), D-fucose (Fuc), D-galacturonic acid monohydrate (GalA), D-mannose (Man), cellulase (EC 3.2.1.4), α-amylase (EC 3.2.1.1), starch (ST) and acetic anhydride were purchased from Sigma (St. Louis, MO, USA). CM-cellulose (CMC), laminaribiose (95%), laminaritriose (95%), and laminaritetraose (95%), laminaripentaose (95%), laminarihexaose (95%), β-1,4-mannanase (EC 3.2.1.78) and konjiac glucomannan (KG) were purchased from Megazyme (Wicklow, Ireland). ANTS (8-aminonaphthalene-1,3,6-trisulfonic acid) was purchased from Tokyo Chemical Industry (Tokyo, Japan). 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 specific polysaccharides in D. huoshanense

All samples were dried by freeze-drying for 48 h, and ground to a fine powder. Each sample (1.0 g) were immersed in 30.0 mL of methanol (80%, *v*/*v*), then refluxed in a Syncore parallel reactor (Büchi, Flawil, Switzerland) for 1.0 h at 65 °C with stirring at 200 rpm. Subsequently, the extract solution was centrifuged at 4000*g* for 10 min (Allegra X-15 centrifuge; Beckman Coulter, Fullerton, CA, USA), the supernatant was removed. The extracted residue was dried under vacuum at 45 °C for 4 h, and water-soluble polysaccharides in *D. huoshanense* were extracted by using microwave assisted extraction according to a previous reported method with modification [19]. Briefly, the dried residues (~1.0 g) were suspended in 60.0 mL of deionized water and extracted with microwave assisted extraction (Multiwave 3000, Anton paar GmbH, Graz, Austria). The microwave irradiation program was performed at 800 W (ramp time 0.5 min) and 80 °C for 9.0 min. Then the extract was centrifuged at 4000*g* for 10 min. The supernatant (~60.0 mL) was evaporated to an appropriate volume under vacuum using rotary evaporator. Subsequently, absolute ethanol was added to the final concentration of 75% (*v*/*v*) for precipitation of crude polysaccharides at room temperature. Then, the sample was centrifuged, and the precipitation was redissolved in 15.0 mL of hot water (60 °C). After centrifugation (4500*g* for 15 min) again, the crude polysaccharides solution was collected and applied for starch analysis by iodine-potassium iodide chromogenic method. Starch was completely removed by α-amylase (20 U/mL) treatment (for 24 h) to no starch colorization of iodine-potassium iodide solution. After centrifugation (4500*g* for 15 min), the supernatant was transferred for ultracentrifugal filtration (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). Finally, the residual specific polysaccharides were lyophilized for further analysis.

2.3. GC–MS analysis

Compositional monosaccharide analysis of specific polysaccharides in *D. huoshanense* was carried out according to the reported method with minor modification [20]. Briefly, the sample of specific polysaccharides (3.0 mg) was hydrolyzed with 2.0 M trifluoroacetic acid (TFA, 1.0 mL), the reaction mixture was performed under microwave irradiation (Multiwave 3000, Anton paar GmbH). The microwave irradiation program was performed at 500 W, 100 °C, for 4 min. After hydrolysis, the hydrolysates were washed with methanol and evaporated to dryness with a nitrogen evaporator for three times to remove the residue of TFA. Subsequently, 0.5 mL of pyridine and 10.0 mg of hydroxylamine hydrochloride were added and incubated at 90 °C for 30 min, then 0.5 mL of acetic anhydride was added and incubated at 90 °C for 30 min. The derivatives were analyzed by using an Agilent 6890 gas chromatography instrument coupled to an Agilent 5973 mass spectrometer (Palo Alto, CA, USA). A capillary column (30 m × 0.25 mm, i.d.) coated with 0.25 μm film 5% phenyl methyl siloxane was used for separation. High purity helium was used as carried gas with a flow rate of 1 mL/min. The column temperature was set at 165 °C and held for 7 min for injection, then programmed at 5 °C/min to 185 °C and held for 5 min, then at 4 °C/min to 200 °C, and finally, at 20 °C/min to 280 °C, and held for 2 min. The split ratio was set as 10:1. The mass spectrometer was operated in electron impact mode, the ionization energy was 70 eV and the scan rate was 0.34 s per scan. The temperatures of ionization source and the transfer line were 150 °C and 280 °C, respectively.

Methylation analysis of specific polysaccharides in *D. huoshanense* was carried out according to the reported method with modification [21]. Briefly, to a solution of specific polysaccharides (3.0 mg in 2.0 mL of DMSO with 20.0 mg of NaOH), 0.15 mL of CH_3I was added. After each addition of the reagents, the reaction mixture was performed under microwave irradiation. The microwave irradiation program was performed at 200 W and 100 °C for 240 s. Finally, the reaction mixture was cooled to room temperature and dialyzed using Spectra/Por Float-A-Lyzer dialysis tube with molecular weight cutoff of 3.5 kDa (Spectrum Labs, Rancho Dominguez, CA, USA) overnight against deionized water, then the reaction mixture was evaporated to dryness with a nitrogen evaporator. Subsequently, the residue was redissolved in 2.0 mL of 2.0 M TFA and exposed to microwave irradiation under 400 W and