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Structural studies of an arabinan from the stems of *Ephedra sinica* by methylation analysis and 1D and 2D NMR spectroscopy

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ABSTRACT

Plant arabinan has important biological activity. In this study, a water-soluble arabinan ($M_w \sim 6.15$ kDa) isolated from the stems of *Ephedra sinica* was found to consist of (1→5)-Ara*f*, (1→3,5)-Ara*f*, T-Ara*f*, (1→3)-Ara*f* and (1→2,5)-Ara*f* residues at proportions of 10:2:3:2:1. A tentative structure was proposed by methylation analysis, nuclear magnetic resonance (NMR) spectroscopy (^{1}H NMR, ^{13}C NMR, DEPT-135, ^{1}H–^{1}H COSY, HSQC, HMBC and ROESY) and literature. The structure proposed includes a branched (1→5)-*α*-Ara*f* backbone where branching occurs at the O-2 and O-3 positions of the residues with 7.7% and 15.4% of the 1,5-linked *α*-Ara*f* substituted at the O-2 and O-3 positions. The presence of a branched structure was further observed by atomic force microscopy. This polymer was characterized as having a much longer linear (1→5)-*α*-Ara*f* backbone as a repeating unit. In particular, the presence of *α*-Ara*f*→3)-*α*-Ara*f*-(1→3)-*α*-Ara*f*-(1→ attached at the O-2 is a new finding. This study may facilitate a deeper understanding of structure–activity relationships of biological polysaccharides from the stems of *E. sinica*.

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1. Introduction

Ephedra sinica Stapf, known as Ma Huang in Chinese, is a shrub used medically for centuries (Mehendale, Bauer & Yuan, 2004). *E. sinica* contains a high account of polysaccharides, which range from 3% to 5% of the total dry weight (Xia et al., 2011b). Recently, polysaccharides from *E. sinica* have been shown to have immunosuppressive effects (Kuang, Xia, Yang, Wang & Wang, 2011; Kuang et al., 2011b). Until recently, various polysaccharides from *E. sinica* have been extensively investigated, including chemical characterization and pharmacological functions widely studied in our lab (Kuang et al., 2011c; Xia et al., 2011a).

During the past decade, an impressive number of structurally diverse and biologically active arabinans have been isolated and identified from herb plants (Cardoso, Silva & Coimbra, 2002; Dourado, Cardoso, Silva, Gama & Coimbra, 2006; Mandal et al., 2011). Furthermore, arabinan and arabinan-rich pectins have been reported to possess a wide range of biological activity profiles including gastro-protective (Cordeiro et al., 2012), as well as immunological (Dourado et al., 2004; Mandal et al., 2013) and anticoagulant activities (Fernandez et al., 2013; Uehara, Takeshita & Maeda, 1992).

As part of the ongoing studies of biological polysaccharides, we isolated a water-soluble arabinan from the stem of *E. sinica* and seek to develop a carbohydrate-based drug. To date, there is no structural study on arabinan from the stem of *E. sinica*. Therefore, it is important to carry out the detailed structural research on this polysaccharide because it will elucidate its structure-bioactivity relationship, which is beneficial for the development of polysaccharides from the stems of *E. sinica*. Detailed structural studies of this arabinan molecule were carried out by methylation analysis, 1D and 2D-NMR experiments (^{1}H, ^{13}C, DEPT-135, ^{1}H–^{1}H COSY, HSQC, HMBC and ROESY) and atomic force microscopy.

2. Materials and methods

2.1. Isolation and purification of ESP-B1H from E. sinica

The ESP-B1 was isolated from the stems of *E. sinica* according to our previous procedure (Kuang et al., 2011a). Fraction ESP-B1 (120.0 mg) was further reiteratively chromatographed over Sephadex G50 eluting with distilled water to afford ESP-B1H (90.0 mg). The yield regarding the wet/dry plant mass was 0.15 mg/g for ESP-B1H.

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2.2. Determination of molecular weight

The molecular weight of the polysaccharide was determined by gel-permeation chromatography. Dextran standards (T-10, T-40, T-70, T-500 and T-2000) were used as a calibration curve by HPLC-ELSD on a Shodex sugar KS-805 column (8.0 mm × 300 mm, 17 μm) coupled with a Shodex KS-G guard column (6 mm × 50 mm, 7 μm). Detailed conditions have been described in our previous report (Kuang et al., 2011c).

2.3. Monosaccharide compositional analysis

The polysaccharide (3 mg) was hydrolyzed with 2 M CF_3COOH in a round bottom flask at 100 °C for 10 h in a boiling water bath. The excess acid was completely removed and co-distilled with methanol. A PMP derivative was made from the hydrolyzed product. The analysis of PMP-labeled monosaccharides was carried out on a Waters Acquity UPLC system coupled with a photodiode array detection (Kuang et al., 2011c).

2.4. Methylation analysis

The polysaccharide ESP-B1H (5 mg) was methylated three times with powdered sodium hydroxide and methyl iodide in dimethy sulphoxide solution. The methylated products were isolated by partitioning between $CHCl_3$ and H_2O (3:1, v/v). The organic layer containing products was washed with 3 mL water three times and dried. The resulting partially methylated products were hydrolyzed, reduced, acetylated and analyzed using a DSQ II GC–MS system (Thermo Fisher Scientific) with DB-5 capillary column (30 m × 0.25 mm × 0.25 μm). The oven condition was initially 80 °C during injection for 1 min. It increased at 5 °C/min to 200 °C, then 10 °C/min to 270 °C and then held at this temperature for 1 min. The partially methylated alditol acetates were identified by their relative retention times on GC and fragment ions in EI-MS, and the molar ratios were estimated from the peak areas.

2.5. Nuclear magnetic resonance (NMR) spectroscopy

The sample ESP-B1H was dissolved in D_2O to exchange the active hydrogen and was then lyophilized. This process was repeated three times. The ^1H NMR and ^{13}C NMR spectra were obtained on a Bruker DPX 400 MHz for δ_H and 100 MHz for δ_C, at room temperature (25 °C), in D_2O. The DEPT experiments were done using a polarization-transfer pulse of 135°. The ROESY mixing delay was 300 ms. The ^1H–^1H COSY, HSQC and HMBC were carried out using standard Bruker software. Chemical shifts are given as δ values with reference to tetramethylsilane (TMS) as an internal standard, and coupling constants are given in Hz.

2.6. Atomic force microscopy (AFM)

The atomic force microscopy in this study was recorded in SPI38OON atomic force microscope (Harbin Institute of Technology, Harbin, China). Atomic force microscopy used tapping mode. The ESP-B1H was diluted to a final concentration of 5.0 μg/mL in distilled water. 300 μL samples were dropped onto freshly cleaved mica and allowed to stand in air before imaging.

3. Results and discussion

3.1. Monosaccharide composition and molecular weight

Although ESP-B1 was detected as a symmetrical peak, some minor impurities were found via Sephacryl S-100 HR column chromatography (Kuang et al., 2011a). Thus, this fraction was further purified on Sephadex G-50 to yield ESP-B1H featured at high arabinofuranosyl content. The ESP-B1H appeared as a highly symmetrical and sharp peak in HPLC-ELSD with Shodex sugar KS-805 column (Fig. 1). Thus, ESP-B1H was selected for structural research on the basis of chemical and extensive spectroscopic analysis.

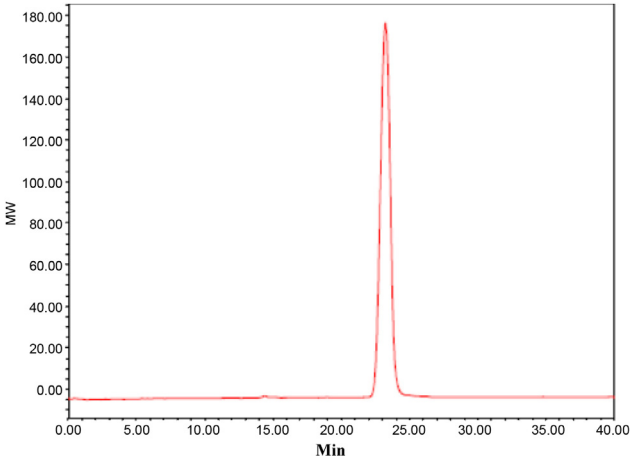

Fig. 1. HPLC-ELSD chromatogram of ESP-B1H using a Shodex sugar KS-805.

The ESP-B1H was a white, amorphous powder. The average molecular weight was 6.15 kDa using standard dextrans. The monosaccharide composition of ESP-B1H was analyzed by UPLC analysis based on pre-column derivatization with PMP reagent after full acid hydrolysis. Compared to monosaccharide standards, ESP-B1H was mainly composed of arabinose (96%) with trace amount of neutral sugars such as glucose, mannose and galactose. This indicated that ESP-B1H was an arabinan.

3.2. Methylation and GC–MS analysis

It is well-known that methylation analysis is the most commonly used method for determining the substitutional pattern of the monosaccharide units in polysaccharides (Kang et al., 2011; Yin, Lin, Nie, Cui & Xie, 2012). The preparation of partially O-methylated alditol acetates (PMAAs) involved successive methylation, hydrolysis, reduction and acetylation identified with GC–MS using EI-MS fingerprints.

As shown in Table 1, five major GC–MS chromatography peaks at 18.05, 20.28, 21.11, 22.77 and 23.08 min can be identified as 2,3,5-tri-O-methyl-1,4-di-O-acetyl-arabinitol, 2,3-di-O-methyl-1,4,5-tri-O-acetyl-arabinitol, 2-O-methyl-1,3,4,5-tri-O-acetyl-arabinitol, 3-O-methyl-1,2,4,5-tri-O-acetyl-arabinitol and 2,5-di-O-methyl-1,3,4-tri-O-acetyl-arabinitol based on characteristic EI-MS fragmentation pathways of different PMAAs, respectively. These data further indicated that all arabinosyl residues should be a furanose rather than a pyranose.

Thus, the typical glycosidic linkages of arabinosyl residues were determined to be T-Ara*f*, (1→5)-Ara*f*, (1→3,5)-Ara*f*, (1→2,5)-Ara*f* and (1→3)-Ara*f* in a molar ratio of nearly 3:10:2:1:2. The semi-qualitative results showed (1→5)-Ara*f* is the most important linkage unit and accounts for more than 55% of the residues. This result indicates this molecule includes a (1→5)-α-Ara*f* backbone where branching occurs at the O-2 and O-3 positions of the residues.

3.3. 1D and 2D NMR analysis

3.3.1. ^1H NMR analysis

The downfield chemical shifts at δ 4.9–5.2 in the ^1H NMR spectrum of ESP-B1H indicated a structural feature of common

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