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Study on *Dendrobium officinale* *O*-acetyl-glucomannan (Dendronan®): Part II. Fine structures of *O*-acetylated residues

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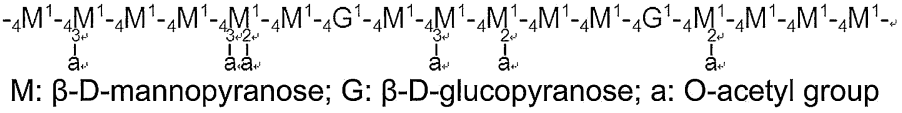
ABSTRACT

Main objective of this study was to investigate the detailed structural information about *O*-acetylated sugar residues in Dendronan®. A water solution (2%, w/w) of Dendronan® was treated with endo-β-mannanase to produce oligosaccharides rich in *O*-acetylated sugar residues. The oligosaccharides were partly recovered by ethanol precipitation (70%, w/w). The recovered sample (designated Hydrolyzed *Dendrobium officinale* Polysaccharide, HDOP) had a yield of 24.7% based on the dry weight of Dendronan® and was highly *O*-acetylated. A D_2O solution of HDOP (6%, w/w) generated strong signals in ^1H, ^{13}C, 2D ^1H–^1H COSY, 2D ^1H–^1H TOCSY, 2D ^1H–^1H NOESY, 2D ^1H–^{13}C HMQC, and 2D ^1H–^{13}C HMBC NMR spectra. Results of NMR analyses showed that the majority of *O*-acetylated mannoses were mono-substituted with acetyl groups at O-2 or O-3 position. There were small amounts of mannose residues with di-*O*-acetyl substitution at both O-2 and O-3 positions. Minor levels of mannoses with 6-*O*-acetyl, 2,6-di-*O*-acetyl, and 3,6-di-*O*-acetyl substitutions were also identified. Much information about sugar residue sequence was extracted from 2D ^1H–^{13}C HMBC and 2D ^1H–^1H NOESY spectra. $^1J_{C-H}$ coupling constants of major sugar residues were obtained. Evidences for the existence of branches or *O*-acetylated glucoses in HDOP were not found. The major structure of Dendronan® is shown as follows:



M: β-D-mannopyranose; G: β-D-glucopyranose; a: O-acetyl group

1. Introduction

Dendrobium officinale plant is an important traditional Chinese herbal medicine and nutraceutical food with many health-enhancing functions (Hua, Zhang, Fu, Chen, & Chan, 2004). The demand for this particular food is so high that more than 130 companies are producing it for commercial purposes in China. Because of the importance of the plant, it is meaningful to understand its chemical components. Previously, we successfully isolated Dendronan®, an 2,3-*O*-acetyl-glucomannan, from dry *D. officinale* herbal samples (Xing et al., 2014). Dendronan® demonstrated a mannose-to-glucose ratio of 6.9:1 and a weight average molecular weight (Mw) of 312 kDa. Methylation analysis indicated that Dendronan® was composed of →4)-Man*p*-(1→ and →4)-Glc*p*-(1→ and did not contain branches. 2D ^1H–^1H COSY and 2D ^1H–^1H TOCSY study indicated the substitution of acetyl groups at O-2 or O-3 position of some mannoses in Dendronan® (Xing et al., 2014). However, the relatively high viscosity of Dendronan® D_2O solution made it difficult to obtain high-resolution heteronuclear 2D ^1H–^{13}C NMR spectra such as 2D ^1H–^{13}C HMBC, 2D ^1H–^{13}C HMQC. Due to the lack of such heteronuclear 2D NMR spectra, only part of the signals from

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major residues of Dendronan® were assigned and more detailed structural information of the sample could not be obtained in the previous study (Xing et al., 2014).

An interesting finding from Xing et al. (2014) was that extensive mannanase hydrolysis of Dendronan® resulted in the production of *O*-acetyl group-rich oligosaccharides that were highly resistant to the enzymatic hydrolysis. This finding inspired us to hypothesize in the current study that the mannanase-resistant oligosaccharides, due to their low-molecular-weight and *O*-acetyl group-rich nature, could be dissolved in D_2O to form a solution with relatively low viscosity and high sample concentration, and such solution could provide full sets of high-resolution 2D NMR spectra with strong signals related to *O*-acetylated residues.

Recovering oligosaccharides from aqueous solutions using ethanol precipitation has been reported by some research groups (Ku, Jansen, Oles, Lazar, & Rader, 2003; Liu, Peng, & Liu, 2012; Price, Hartman, Faber, Vermillion, & Fahey, 2011; Roxas, Fukuba, & Mendoza, 1985; Sen et al., 2011; Swennen, Courtin, Van der Bruggen, Vandecasteele, & Delcour, 2005; Thurl, Offermanns, Müller-Werner, & Sawatzki, 1991; Xu, Lin, & Shi, 2011; Zhang, Zhang, & Wang, 2009). For example, using 50% (v/v) ethanol precipitation, Price et al. (2011) successfully recovered galactoglucomannan oligosaccharides from an acid-hydrolyzed molasses byproduct of pine fiberboard production. Although this method has the drawback of incomplete precipitation of oligosaccharides, it is simple and quick in obtaining relatively large amounts of sample (Ku et al., 2003). Therefore, in the current study, we attempted to use ethanol precipitation to collect oligosaccharides resulting from the hydrolysis of Dendronan® by mannanase, which was followed by obtaining a set of high-resolution 2D NMR spectra of the collected sample and extracting detailed structural information regarding the *O*-acetylated sugar residues and glycosidic linkages from the spectra obtained.

2. Materials and methods

2.1. Sample preparation

Dendronan® was extracted and purified as described in our previous report (Xing et al., 2014). The complete dissolution of Dendronan® (2 g) in 100 mL of de-ionized water in a 500 mL beaker was achieved by stirring and heating at 70 °C for 6 h, and then temperature of the solution was allowed to drop to 37 °C. Subsequently, 50 μL of endo-β-1,4-mannanase suspension (*Aspergillus niger*, Megazyme, Ireland,. 297 U/mL) was added to the solution, followed by stirring at 37 °C for 40 min. Immediately following the 40-min enzymatic treatment, the beaker was put in a 95 °C water bath with stirring for 20 min to deactivate the enzyme and then was allowed to cool at room temperature. Ethanol (31 mL) pre-cooled at 4 °C was added dropwise to the solution with vigorous stirring to form a mixture with an ethanol concentration of around 20% (w/w). After staying at 4 °C for 6 h, the mixture was centrifuged at 10,000 × *g* and 4 °C for 15 min. The minor amount of precipitate was discarded and the supernatant was transferred to a 500 mL beaker. Subsequently, 260 mL of 100% ethanol pre-cooled at −20 °C was added dropwise to the supernatant with vigorous stirring to form a suspension with an ethanol concentration of approximately 70% (w/w). The system was then kept at −20 °C for 12 h to facilitate the formation of precipitate, after which the precipitate was collected by centrifuge at 10,000 × *g* and 4 °C for 15 min. The collected precipitate was re-dissolved in de-ionized water (50 mL), and the resultant solution was frozen and freeze-dried to produce a dry sample (designated HDOP). HDOP was then subjected to Maldi-tof-MS analysis to obtain information about the distribution of molecular weight, according to Xing et al. (2014).

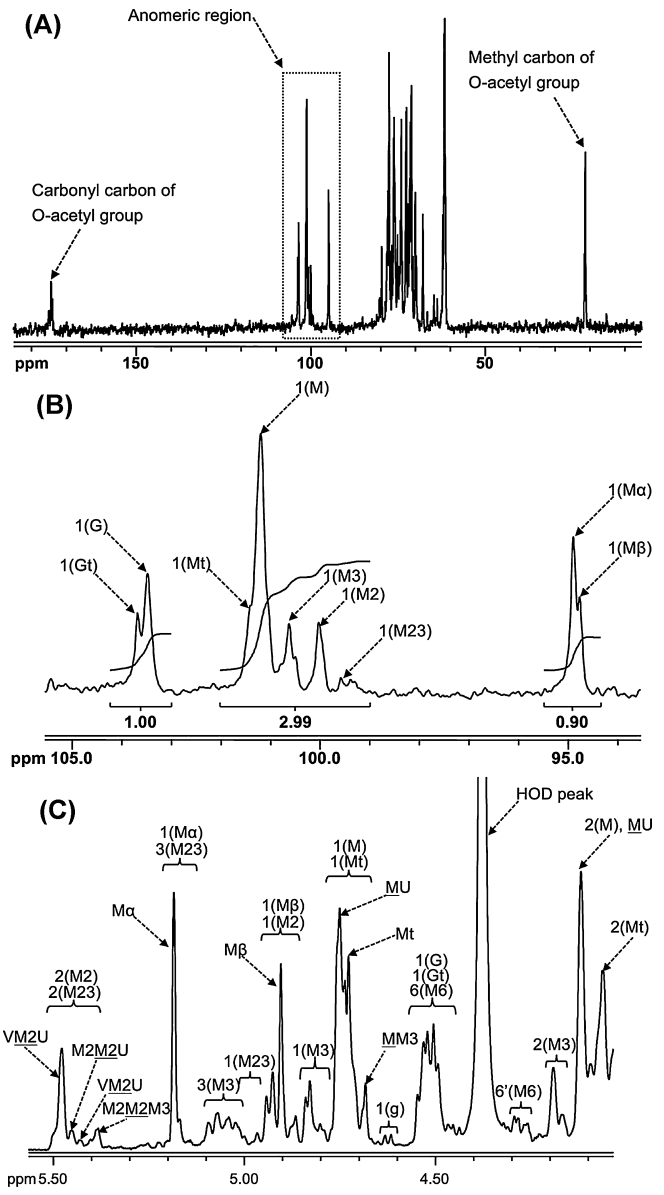

Fig. 1. 1D ^{13}C spectrum ((A) and (B)) and ^{1}H NMR spectrum (C) of HDOP. "U" means residues G, M, or M2 in the reducing end side. "V" means residue G, M, or M3 in the non-reducing end side. For example, 1(G) in figure (B) and figure (C) means the signal comes from the C-1 and H-1 of glucose residue G, respectively. 1(g) denotes the signal from H-1 of galactose residue (designated residue g) of β-(1 → 4)-D-galactan contaminant.

2.2. 1D and 2D NMR spectroscopy

The sample for 1D and 2D NMR spectroscopy was prepared by dissolving around 240 mg of HDOP in 5 mL of D_2O and then freeze-drying the solution; this dissolution-drying process was repeated three times. The sample recovered from the third freeze-drying treatment was re-dissolved in 4 mL of D_2O, passed through a nylon syringe filter (pore size 1.5 μm), and transferred to a regular NMR tube (5 mm). The ^{1}H and ^{13}C NMR spectra of the sample were acquired, respectively, at 500.13 and 125.78 MHz on a Bruker AMX 500FT NMR spectrometer (Bruker Co., Germany). A $^{1}H/^{13}C/^{15}N$ probe with 5-mm inverse geometry was used. Trimethylsilyl propionate (TSP) in D_2O and 1,4-dioxane in D_2O were used as ^{1}H and ^{13}C NMR chemical shift standards, respectively. 1D ^{1}H NMR experiment, 1D ^{13}C NMR experiment, and a series of 2D NMR experiments including 2D ^{1}H–^{1}H correlation NMR spectroscopy (COSY), 2D