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Isolation and chemical characterization of a glucogalactomannan of the medicinal mushroom *Cordyceps militaris*

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ABSTRACT

Cordyceps militaris dried fruiting bodies were extracted with 5% KOH solution. The extract was purified by freeze-thawing treatment, and dialysis (100 kDa), giving rise to a homogeneous polysaccharide (M_w 23,000 Da). Its monosaccharide composition was mannose (56.7%), galactose (34.5%), and glucose (8.8%). The anomeric configurations were determined by their coupling constants. A complex polysaccharide was identified by NMR and methylation analysis. The HSQC spectrum showed signals at δ 107.7/5.06 and 106.1/5.14; 105.9/5.12 relative to β-D-Gal*f*, and O-2-substituted β-D-Gal*f* units, respectively. The sign at δ 104.4/5.21 corresponded to α-D-Gal*f*. Other signals corresponded to α-D-Man*p* O-6- and O-2-substituted (δ 100.2/4.94; 100.5/5.27; 100.6/5.23; 100.7/5.16), and α-D-Man*p* 2,6-di-*O*-substituted (from δ 99.3 to 99.9). The main linkages, confirmed by methylation analysis, showed the derivatives: 2,3,4-Me_3-Man*p* (11.9%) and 3,4,6-Me_3-Man*p* (28.6%). The branches were (1 → 6)-linked-α-D-Man*p* or (1 → 2)-linked-β-D-Gal*f*, terminating with β-D-Gal*f*, α-D-Gal*f*, α-D-Gal*p*, or α-D-Man*p*. 42.7% of the partially hydrolyzed product consisted of 3,4,6-Me_3-Man*p*, suggesting a (1 → 2)-linked backbone.

1. Introduction

The scientific community have provided plenty of data showing that mushroom extracts demonstrate interesting biological properties such as antitumor (Daba & Ezeronye, 2003), antiinflammatory (Komura, Carbonero, et al., 2010), antiviral (Lindequist, Niedermeyer, & Jülich, 2005), and immunomodulatory effects (Chanput et al., 2012; Chen, Zhang, Shen, & Wang, 2010; Lin et al., 2012; Smiderle et al., 2011). These extracts may contain different molecules as steroids, polyphenols, hydroquinones, triterpenes, ubiquitin-like proteases, anti-protozoal compounds, proteins, glycoproteins, and polysaccharides that are involved in such biological effects (Lindequist et al., 2005).

Several mushrooms have been studied for their pharmacological potentials. Among them, *Cordyceps militaris*, an entomopathogenic fungus belonging to the class Ascomycetes, is the one of the most important traditional Chinese medicines, being the second most commercialized species in China, Korea, and Japan (Das, Masuda, Sakurai, & Sakakibara, 2010; Wang et al., 2012). *C. militaris* is used as a folk tonic in East Asia and the studies related to its pharmacological properties suggest that this mushroom can exert antioxidant, antiviral, and immuno-protective activities (Ohta, Lee, Hayashi, & Fujita, 2007; Wang et al., 2012; Yu et al., 2009). It contains many active components such as cordycepin, ergosterol, mannitol, and polysaccharides, as β-glucans (Holliday & Cleaver, 2008).

Most of the benefits provided by mushroom extracts can be attributed to their polysaccharides, which have attracted much attention in the past 50 years (Ren, Perera, & Hemar, 2012). These polymers, also known as "biological response modifiers (BRMs)," are able to stimulate the innate immune system and to promote the stimulation of the host's defence mechanism, exerting antitumoral, antiviral, and antimicrobial activity (Lindequist et al., 2005; Ramberg, Nelson, & Sinnott, 2010; Schepetkin & Quinn, 2006).

The most studied mushroom polysaccharides are β-glucans, which are vastly isolated from Basidiomycetes (Ren et al., 2012; Smiderle et al., 2006), and present plenty of biological activities (Baggio et al., 2012; Chen & Seviour, 2007; Smiderle et al., 2013). However also heteropolysaccharides are encountered in mushrooms, showing interesting therapeutic importance (Zhang, Cui, Cheung, & Wang, 2007). The most common heteropolysaccharides isolated from Basidiomycetes are heterogalactans, as mannogalactans (Smiderle et al., 2008), fucogalactans (Komura, Carbonero, et al., 2010), and mannofucogalactans (Zhang et al., 2007). The Ascomycetes present mainly heteropolysaccharides based on D-mannan main chains as glucomannans and galactomannans (Barreto-Bergter & Gorin, 1983). The chemical structure of these heteropolymers is more complex than the β-glucans. There are considerable studies on these molecules because they can

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be used for different purposes as taxonomic classification of the species (Carbonero, Mellinger, Eliasaro, Gorin, & Iacomini, 2005) and for treatment of various diseases (Ren et al., 2012).

Elucidation of the chemical structure of polysaccharides has shown to be challenging, considering that these molecules can present different monosaccharide composition, linkages, α/β-configuration, and molecular weight, and that this chemical characteristic can influence their bioactivity (Lehtovaara & Gu, 2011). Generally, the greater the molecular weight and the higher the water solubility of the polysaccharides, the higher the antitumor activity (Daba & Ezeronye, 2003). A study based on seven potent antitumor polysaccharide–protein complexes from *Ganoderma tsugae*, has found that heteropolysaccharides, with M_w of about 10,000 Da, containing galactose, glucose, mannose, and fucose, showed the highest antitumoral activity (Ren et al., 2012).

Besides, the polysaccharides can assume distinct quaternary structures, such as single or multiple-helices. For instance, lentinan, the β-D-glucan isolated from Shiitake (*Lentinus edodes*), in its triple-helical conformation, was found to inhibit the growth of solid tumours (sarcoma-180) in mouse. This inhibitory effect was not observed when the mice were treated with the single-helical polysaccharide (Zhang, Li, Xu, & Zeng, 2005).

Polysaccharides belong to a structurally diverse class of macromolecules. The monosaccharide units of these polymers can interconnect at several points to produce various branched or linear structures. This vast potential variability in carbohydrate structures could offer possibilities to the precise regulatory mechanisms of various cell–cell interactions in higher organisms. All this information shows how important it is to define the chemical structure of the biologically active polysaccharides. Chemical characterization of these molecules is required to provide enough data for the elucidation of their biological effects. Considering this, the aim of the present study was to isolate, purify and chemically characterize a complex heteropolysaccharide from *C. militaris*.

2. Experimental

2.1. Fungal material

Fruiting bodies of *C. militaris* (L.) Link (strain: MCI 10304, Mushtech Cordyceps Institute) were a kind gift from Dr. J. M. Sung of Kangwon National University (Chuncheon, Korea).

2.2. Extraction and purification of the heteropolysaccharide

The dried mushroom (28.7 g) was submitted to several extraction steps as shown in Fig. 1. Briefly, the material was firstly treated with $CHCl_3$:MeOH (1:1, v/v), using a Soxhlet, under heating (50 °C), for 3 days. After removing the apolar compounds and the excess of solvents, the residue I was successively submitted to cold (25 °C) and hot (100 °C) aqueous extractions (for 6 h, 3× for each extraction). The aqueous extracts were used for other studies. The remaining residue III was extracted twice with 5% KOH solution at 100 °C, for 6 h, giving rise to an alkaline extract (K5), which was neutralized with glacial acetic acid and dialysed (12–14 kDa), for 24 h. The extract (K5) was solubilized in water and submitted to freezing followed by mild thawing at 4 °C (Gorin & Iacomini, 1984). This process was repeated 5× to guarantee a complete separation of the water-soluble (SK5) of the non-soluble (PK5) polysaccharides. Both fractions were separated by centrifugation (12,000 rpm, at 4 °C, for 20 min), and freeze-dried. The soluble fraction (SK5) was the focus of this study, after a treatment with dimethylsulfoxide (50 mL), for 30 min, at 50 °C. The Me_2SO-insoluble material (PD-SK5) was recovered by centrifugation (10,000 rpm, at 20 °C, for 15 min) and dialysed against tap water

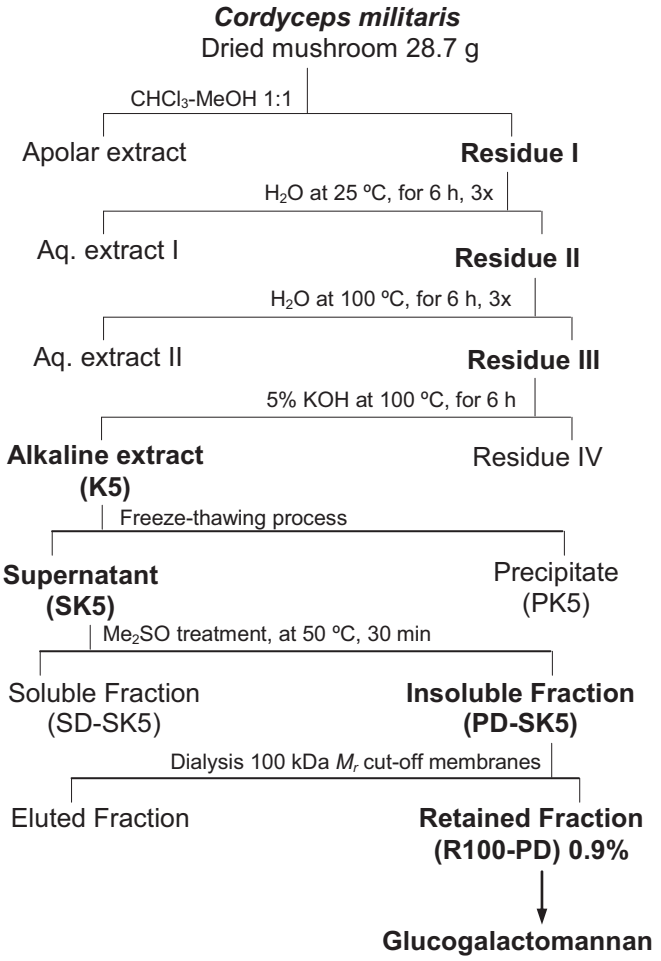

Fig. 1. Extraction and purification steps of the glucogalactomannan (R100-PD) of *C. militaris*.

for 24 h, to remove the solvent. This fraction was dialysed (100 kDa, cut-off) against distilled water, giving rise to a purified polysaccharide, which was retained by the dialysis membrane (R100-PD).

2.3. Alditol acetates preparation for monosaccharide composition analysis

Each polysaccharide fraction (1 mg) was hydrolyzed with 2 M TFA at 100 °C for 8 h, followed by evaporation to dryness. The dried carbohydrate samples were dissolved in 0.5 N NH_4OH (100 μL), held at room temperature for 10–15 min in reinforced 4 ml Pyrex tubes with Teflon lined screw caps. $NaBH_4$ (1 mg) was added, and the solution was kept at 100 °C for 10 min, in order to reduce aldoses to alditols (Sassaki et al., 2008). The excess of $NaBH_4$ was neutralized by the addition of acetic acid (30 μL), and removed by the addition of methanol (×2) under a N_2 stream in a fume hood. The reduced product was dried and acetylation of the Me-alditols was performed in pyridine–Ac_2O (200 μL; 1:1, v/v), for 30 min at 100 °C. The resulting alditol acetates were analyzed by GC–MS, and the sugars were identified by their typical retention times and electron impact profiles (Sassaki, Gorin, Souza, Czelusniak, & Iacomini, 2005a).

2.4. Methylation analysis

Per-*O*-methylation of the polysaccharides was carried out using the modified method of Ciucanu and Kerek (1984). An aliquot of each dried polysaccharide (10 mg) was completely solubilized