Carbohydrate Polymers 97 (2013) 74–80

Contents lists available at SciVerse ScienceDirect

Carbohydrate Polymers

journal homepage: www.elsevier.com/locate/carbpol

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

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ARTICLE INFO

Article history:
Received 14 March 2013
Received in revised form 11 April 2013
Accepted 12 April 2013
Available online 24 April 2013

Keywords:
Cordyceps militaris
Glucogalactomannan
Ascomycete
NMR spectrometry

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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0144-8617/$ – see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.carbpol.2013.04.049

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

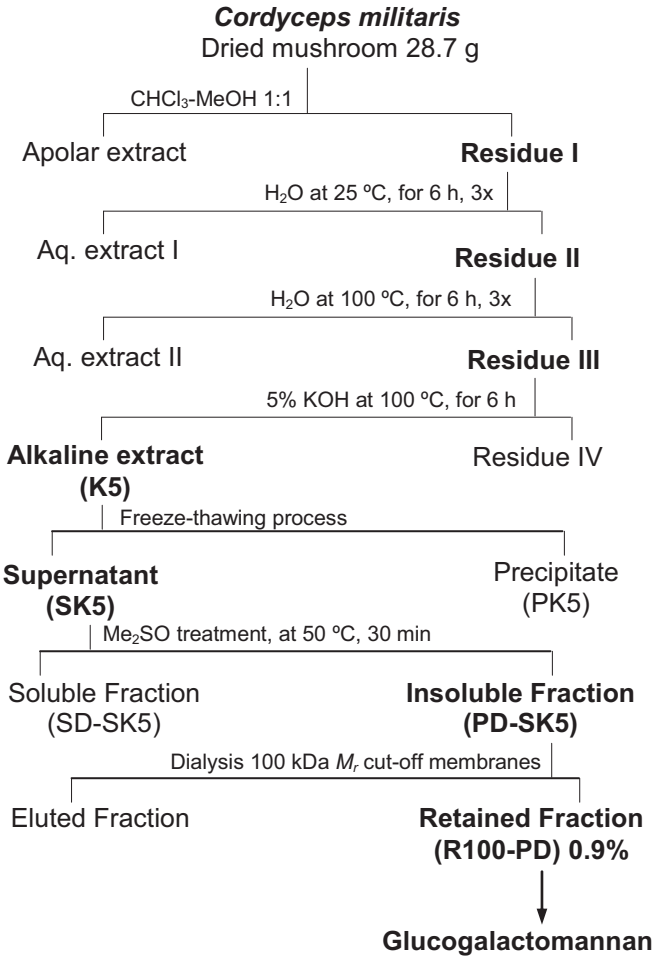

Fig. 1. Extraction and purification steps of the glucogalactomannan (R100-PD) of *C. militaris*.

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

in dimethylsulfoxide (Me_2SO, 1 mL), followed by the addition of iodomethane (MeI, 1 mL) and powdered NaOH (20 mg) (Ciucanu & Kerek, 1984). The mixture was stirred for 30 min or until it turned to solid phase, which was left to react for 14–18 h. The material was re-solubilized in water, on ice, and neutralized with glacial acetic acid. After dialysis (8 kDa) against tap water to remove the excess of salts, the partially *O*-methylated polysaccharides were freeze-dried and the methylation procedure was repeated to guarantee a complete methylation of the free hydroxyls. After this step, the sample was partitioned between chloroform (3 mL) and distilled water (3 mL). The chloroform phase, containing the per-*O*-methylated derivatives, was evaporated and submitted to hydrolysis. The aqueous phase, containing salts and residues of the reaction was discarded. The hydrolysis was proceeded as follows: an aliquot of the dried per-*O*-methylated derivatives was treated with 3% MeOH-HCl (1 mL), for 2 h, at 80 °C, followed by evaporation under N_2. The methanolized material was then submitted to hydrolysis with 1 M H_2SO_4 (1 mL), for 9 h or 12 h, at 100 °C.

After this step the samples were reduced by NaB_2H_4 and acetylated as above (alditol acetates preparation), to give a mixture of partially *O*-methylated alditol acetates, which was analyzed by GC–MS using a DB-225 capillary column as described above. The derivatives were identified from m/z of their positive ions, by comparison with standards, and the results were expressed as relative percentage of each component (Sassaki, Gorin, et al., 2005).

2.5. Gas chromatography–mass spectrometry (GC–MS) analysis

The analyses were performed using a Varian (model 3300) gas chromatograph linked to a Finnigan Ion-Trap model 810 R-12 mass spectrometer, with He as carrier gas. A capillary column (30 m × 0.25 mm i.d.) of DB-225, held at 50 °C during injection and then programmed at 40 °C/min to 220 °C or 210 °C (constant temperature) was used for qualitative and quantitative analysis of alditol acetates and partially *O*-methylated alditol acetates, respectively (Sassaki, Gorin, et al., 2005).

2.6. Nuclear magnetic resonance spectroscopy

NMR spectra (^{1}H-, ^{13}C-, and DEPT-^{13}C-NMR, COSY, TOCSY, NOESY, HSQC, coupled HSQC) were obtained using a 400 MHz Bruker Avance III spectrometer with a 5 mm inverse probe. The analyses were performed at 50 or 70 °C in D_2O, and the chemical shifts are expressed in δ ppm relative to external standard of acetone at δ 30.2 (^{13}C) and 2.22 (^{1}H).

2.7. Determination of homogeneity and molar mass

The homogeneity and molar mass of the purified glucogalactomannan was determined by high performance steric exclusion chromatography (HPSEC), using a refractive index (RI) detector. The eluent was 0.1 M $NaNO_3$, containing 0.5 g/L NaN_3. The solutions were filtered through a membrane of 0.22 μm pore size (Millipore). The molar mass of the polymer was estimated using Astra software 4.70.

2.8. Partial hydrolysis

An aliquot of the fraction R100-PD (100 mg) was partially hydrolyzed with aqueous trifluoroacetic acid (TFA), adjusted to pH 2.0 (1 ml), at 100 °C, for 12 h. A polymeric product (PH) was obtained by precipitation with EtOH (3:1; v/v) from a small volume of water, and then retained on a dialysis membrane (2 kDa cut-off). The PH fraction was lyophilized and analyzed by NMR spectrometry and GC–MS.

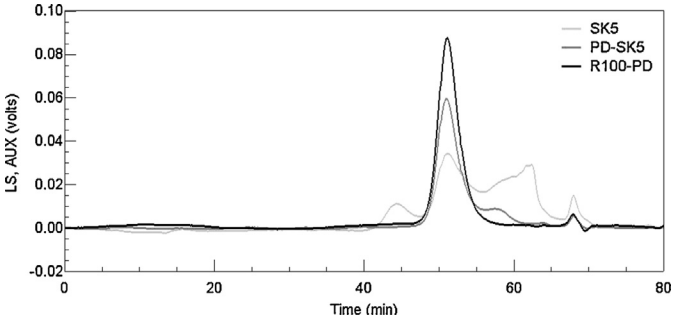

Fig. 2. Elution profiles of fractions SK5, PD-SK5, and R100-PD determined by HPSEC using refractive index detectors, and 0.1 M $NaNO_3$ as eluent.

3. Results and discussion

The steps of the polysaccharide extraction are shown in Fig. 1. The yield of each fraction was calculated on the basis of initial weight of dried mushroom (28.7 g). The material was firstly treated with $CHCl_3$:MeOH (1:1, v/v), as described in Section 2.2, and contained 3% apolar compounds and 97% residue I. The latter was used for the following extractions. The cold (3.5%) and hot (1.1%) water extracts were not used in the present study. The remaining residue III, after being extracted with 5% KOH solution, presented an extract of 9.8% yield (K5), which was submitted to freeze-thawing for several times, and centrifugation to separate the soluble polysaccharides from the non-soluble ones. This procedure gave rise to a fraction (SK5, 4.9%), that consisted of mannose (37.0%), galactose (14.8%), and glucose (48.2%); its HPSEC analysis showed an heterogeneous profile (Fig. 2). Considering that α- and/or β-glucans are present in mushrooms in great amounts (Schepetkin & Quinn, 2006), this fraction was treated with Me_2SO, which is able to solubilize mainly β-glucans, and after centrifugation, the Me_2SO-insoluble fraction (PD-SK5) was recovered showing a considerable reduction in the amount of glucose (9.5%), compared to mannose and galactose (Table 1). Its HPSEC profile confirmed the reduction of glucose by the absence of peaks at ~45 min and after 60 min (Fig. 2). This fraction still presented a small peak at ~58 min, which was removed after a dialysis on membrane of 100 kDa cut-off. The retained polysaccharide on this membrane (R100-PD) showed 0.9% yield and had a homogeneous profile (Fig. 2). The monosaccharide composition of the purified R100-PD showed mannose (56.7%), galactose (34.5%), and glucose (8.8%), suggesting the presence of a heteropolysaccharide (Table 1). The molecular weight of this molecule was estimated at 23,000 Da.

In order to characterize the glycosidic linkages of the isolated heteropolysaccharide, methylation analysis was performed and the results are shown in Table 2. The analysis showed a complex and branched polysaccharide by the presence of non-reducing end units, as 2,3,4,6-Me_4-Man*p* (1.3%), 2,3,5,6-Me_4-Gal*f* (11.5%), and 2,3,4,6-Me_4-Gal*p* (10.9%), and the derivatives 2,3-Me_2-Glc*p* (9.1%), and 3,4-Me_2-Man*p* (14.9%). The main chain is probably composed of (1 → 2)-linked-mannopyranose, which can be concluded from

Table 1
Monosaccharide composition of the fractions obtained from *C. militaris*.

Fractions	Yield%	Monosaccharides (mol%)[a]		
		Man	Gal	Glc
SK5	4.9	37.0	14.8	48.2
PD-SK5	1.2	58.4	32.1	9.5
R100-PD	0.9	56.7	34.5	8.8
PH	–	84.7	10.4	4.9

[a] Alditol acetates obtained on successive hydrolysis, $NaBH_4$ reduction, and acetylation, followed by GC–MS analysis.

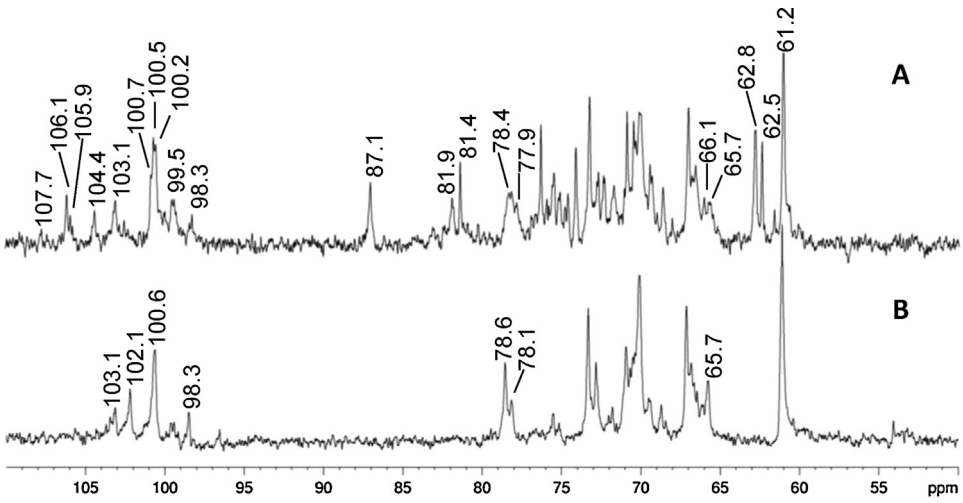

Fig. 3. ^{13}C-NMR spectra of native (R100-PD) (A) and partially degraded (PH) (B) glucogalactomannan, in D_2O at 50 °C (chemical shifts are expressed in ppm).

the presence of high amounts of 3,4,6-Me$_3$-Man*p* (28.6%). According to the methylation data, the branching points may be situated mainly at O-6 position of mannose residues and there is also the presence of (1 → 6)-linked-Man*p* fragments, which was shown by the presence of the derivative 2,3,4-Me$_3$-Man*p* (11.9%).

Galactomannans have been isolated from fungus (Barreto-Bergter & Gorin, 1983; Giménez-Abián, Bernabé, Leal, Jiménez-Barbero, & Prieto, 2007) and lichens (Carbonero, Tischer, Cosentino, Gorin, & Iacomini, 2003; Gorin & Iacomini, 1985) by alkaline extractions and such polymers have shown a great variety of side-chain structures. These heteropolysaccharides presented mainly (1 → 6)-linked-α-D-Man*p* as main chain O-2-substituted by α-D-Gal*p*-(1 → 2)-α-D-Man*p* fragments (Barreto-Bergter & Gorin, 1983; Carbonero et al., 2003). The side-chains may also be substituted at O-4 by α-D-Gal*p*, α-D-Man*p*, and β-D-Gal*f* (Gorin & Iacomini, 1985). A different structure was proposed for the galactomannan of the fungus *Lineolata rhizophorae*, that presented a main chain of (1 → 6)-linked-α-D-Man*p*, substituted at O-3 by non-reducing ends of β-D-Gal*f* or by β-D-Gal*f*-(1 → 5)-β-D-Gal*f* disaccharides (Giménez-Abián et al., 2007).

NMR analysis [^{1}H, ^{13}C (Fig. 3), HSQC (Fig. 4), coupled HSQC, COSY, TOCSY, and NOESY] contributed to elucidate the structure of the heteropolysaccharide. The main signals, which are summarized in Table 3, were assigned according to the correlation among the NMR spectra and to literature values of similar polysaccharides. The α or β-configurations were determined by the coupling constants ($J_{C1/H1}$) of each signal, being α for ~170–175 Hz and β for values ~160 Hz (Perlin & Casu, 1969), except for the furanoside units that do not follow this pattern (Chambat, Joseleau, Lapeyre, & Lefebvre, 1978; Sassaki, Iacomini, & Gorin, 2005b).

The signs of C1/H1 at low field at δ 105.9/5.12; 106.1/5.14; and 107.7/5.06 confirmed the presence of β-galactofunarose 2-*O*-substituted and as non-reducing end units, respectively (Barreto-Bergter & Gorin, 1983; Cordeiro et al., 2005; Giménez-Abián et al., 2007). The sign (C1/H1) at δ 104.4/5.21, showed the presence of terminal α-galactofunarose, and its α-configuration was confirmed by the $J_{C1/H1}$ at 174 Hz (Chambat et al., 1978; Prieto, Leal, Bernabé, & Hawksworth, 2008; Sassaki, Iacomini, et al., 2005). The high field signals of C1/H1 at δ 98.3/5.05 could be attributed to the 2-*O*-substituted and non-reducing end α-galactopyranosyl units (Gorin & Iacomini, 1985; Perry & MacLean, 2004; Smiderle et al., 2008).

The C2/H2 of the 2-*O*-substituted β-Gal*f* units was shown by a shift to 87.1/4.21 ppm, while the C2/H2 of the non-reducing end β-Gal*f* was at δ 81.4/3.83 (Ahrazem et al., 2006; Cordeiro et al., 2005).

Signals (C1/H1) relative to the main chain of (1 → 2)-linked-α-D-Man*p* were observed at δ 100.5/5.27, 100.6/5.23, and 100.7/5.06, while the sign of (1 → 6)-linked-α-D-Man*p* fragments was at δ

Table 2
Partially *O*-methylalditol acetates formed on methylation analysis of native (R100-PD) and partially degraded (PH) glucogalactomannan isolated from *C. militaris*.

Partially *O*-methylated alditol acetates[a]	Mol% R100-PD	Mol% PH[b]	Linkage type[c]
2,3,4,6-Me$_4$-Man*p*	1.3	3.5	Man*p*-(1→
2,3,4,6-Me$_4$-Glc*p*	–	2.4	Glc*p*-(1→
2,3,5,6-Me$_4$-Gal*f*	11.5	–	Gal*f*-(1→
2,3,4,6-Me$_4$-Gal*p*	10.9	7.9	Gal*p*-(1→
3,4,6-Me$_3$-Man*p*	28.6	42.7	2→)-Man*p*-(1→
3,5,6-Me$_3$-Gal*f*	3.8	–	2→)-Gal*f*-(1→
2,3,4-Me$_3$-Man*p*	11.9	30.7	6→)-Man*p*-(1→
3,4,6-Me$_3$-Gal*p*	8.0	–	2→)-Gal*p*-(1→
2,3-Me$_2$-Glc*p*	9.1	5.9	4,6→)-Glc*p*-(1→
3,4-Me$_2$-Man*p*	14.9	6.1	2,6→)-Man*p*-(1→

[a] Analyzed by GC–MS, after methylation, total acid hydrolysis, reduction with $NaBD_4$ and acetylation.
[b] R100-PD after the partial hydrolysis.
[c] Based on derived *O*-methylalditol acetates.

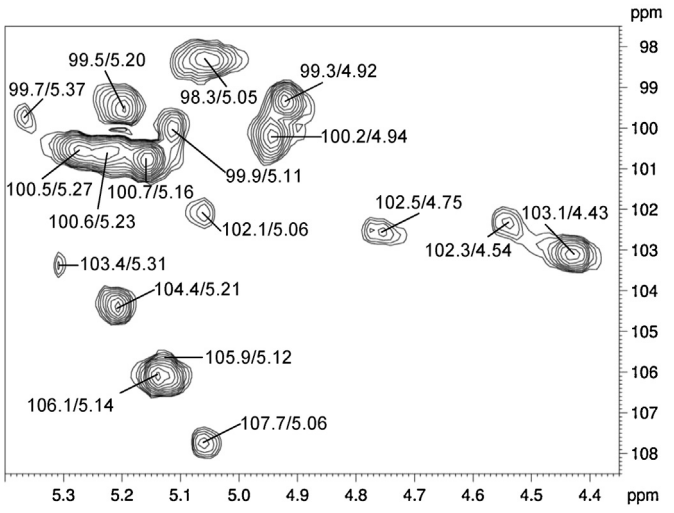

Fig. 4. Anomeric region of HSQC spectrum of glucogalactomannan (R100-PD), in D_2O at 50 °C (chemical shifts are expressed in ppm).

Table 3
^{1}H and ^{13}C NMR chemical shifts of the *C. militaris* glucogalactomannan, and coupling constants ($J_{C1/H1}$) of C1/H1. Assignments are based on ^{13}C, ^{1}H, DEPT, COSY, HSQC, and coupled HSQC analysis.

Units	^{13}C sign[a]	^{1}H sign[a]	$J_{C1/H1}$ (Hz)	References[b]
C1/H1 of β-D-Gal*f*-(1→	107.7	5.06	174	a, b, c
C-1/H-1 of →2)-β-D-Gal*f*-(1→	106.1	5.14	173	b
C-1/H-1 of →2)-β-D-Gal*f*-(1→	105.9	5.12	173	b
C-1/H-1 of α-D-Gal*f*-(1→	104.4	5.21	174	d, e, f
C-1/H-1 of →4,6)-β-D-Glc*p*-(1→	103.1	4.43	161	g, h
C-1/H-1 of →4,6)-β-D-Glc*p*-(1→	102.5	4.75	165	g, h
C-1/H-1 of →4,6)-β-D-Glc*p*-(1→	102.3	4.54	159	g, h
C1/H1 of α-D-Man*p*-(1→	102.1	5.06	173	i
C1/H1 of →2)-α-D-Man*p*-(1→	100.7	5.16	176	j, k
C1/H1 of →2)-α-D-Man*p*-(1→	100.6	5.23	171	j, k
C1/H1 of →2)-α-D-Man*p*-(1→	100.5	5.27	172	j, k
C1/H1 of →6)-α-D-Man*p*-(1→	100.2	4.94	170	j, k, l
C1/H1 of →2,6)-α-D-Man*p*-(1→	99.9	5.11	175	m
C1/H1 of →2,6)-α-D-Man*p*-(1→	99.7	5.37	173	j, k, n
C1/H1 of →2,6)-α-D-Man*p*-(1→	99.5	5.20	172	j, k, n
C1/H1 of →2,6)-α-D-Man*p*-(1→	99.3	4.92	171	j, k, n
C1/H1 of α-D-Gal*p*-(1→	98.3	5.05	173	n, o, p
C-2/H-2 of →2)-β-D-Gal*f*-(1→	87.1	4.21	–	q
C-4/H-4 of β-D-Gal*f*-(1→	81.9	4.03	–	b
C-2/H-2 of β-D-Gal*f*-(1→	81.4	3.83	–	b
C-2 of →2)-α-D-Man*p*-(1→ and →2,6)-α-D-Man*p*-(1→	78.4	4.03	–	c
C-2 of →2)-α-D-Man*p*-(1→ and →2,6)-α-D-Man*p*-(1→	77.9	4.12	–	c
C-6/H-6 of →4,6)-β-D-Glc*p*-(1→	66.1	3.89/3.99	–	g
C-6/H-6 of →6)-α-D-Man*p*-(1→ and →2,6)-α-D-Man*p*-(1→	65.7	3.78/4.00	–	i
C-6/H-6 of β-D-Gal*f*-(1→	62.8	3.71/3.85	–	r
C-6/H-6 of β-D-Gal*f*-(1→	62.5	3.64/3.79	–	r
C-6/H-6 of α-D-Man*p*-(1→ and →2)-α-D-Man*p*-(1→	61.2	3.79/3.88	–	i

[a] The chemical shifts are expressed as ppm (δ).
[b] In accordance with references: a, Giménez-Abián et al., 2007; b, Cordeiro et al., 2005; c, Barreto-Bergter & Gorin, 1983; d, Chambat et al., 1978; e, Sassaki, Iacomini, et al., 2005; f, Prieto et al., 2008; g, Mandal et al., 2012; h, Cui et al., 2000; i, Komura, Ruthes, et al., 2010; j, Viccini et al., 2009; k, Carbonero et al., 2003; l, Rosado et al., 2002; m, Omarsdottir et al., 2006; n, Gorin & Iacomini, 1985; o, Smiderle et al., 2008; p, Perry & MacLean, 2004; q, Ahrazem et al., 2006; r, Gorin & Mazurek, 1975.

100.2/4.94. The 2,6-di-*O*-substitution of the α-D-Man*p* residues was confirmed by the presence of signals from δ 99.3 to 99.9 ppm.

The O-2 and O-6 substitutions of α-D-Man*p* units were confirmed by the presence of signals from δ 77.8 to 78.4 (C2) (Barreto-Bergter & Gorin, 1983), and 65.7 (C6) (Komura, Ruthes, et al., 2010).

Different from lichen and yeast galactomannans, the isolated heteropolysaccharide of *C. militaris* showed a low amount of glucose (8.8%) in its structure. This was observed from the methylation analysis, by the presence of the derivative 2,3-Me$_2$-Glc*p* (9.1%), that was confirmed by the HSQC signals (C1/H1) at δ 102.3/4.54; 102.5/4.75; and 103.1/4.43 ppm (Cui, Wood, Blackwell, & Nikiforuk, 2000; Mandal et al., 2012). The $J_{C1/H1}$ of these units was ~160–165 Hz, which confirmed the β-configuration. The substituted C6 of these units was observed at δ 66.1 ppm.

These data suggested that *C. militaris* alkaline extract contains a glucogalactomannan, with a main chain of (1 → 2)-linked-α-D-Man*p*, that can be substituted at O-6 by (1 → 6)-linked-α-D-Man*p* or (1 → 2)-linked-β-D-Gal*f* fragments, terminating with α-D-Man*p*, α-D-Gal*p*, α-D-Gal*f*, or β-D-Gal*f* units. COSY and NOESY NMR correlations have also indicated that 4,6-di-*O*-substituted-β-D-Glc*p* is present in the structure, probably linked to the main chain as a fragment similar to α-D-Man*p*-(1 → 2)-α-D-Man*p*-(1 → 6)-β-D-Glc*p*-(1 → . The β-D-Glc*p* of this trisaccharide may be also substituted at O-4 by non-reducing end units as α-D-Gal*p*, α-D-Gal*f* or β-D-Gal*f*. The other correlations were not possible because of the large amount of signals at the region from 70 to 80 ppm.

Similar polysaccharides were isolated from *C. militaris* mycelia (Lee, Kwon, Yun, et al., 2010) and from the liquid culture broth (Lee, Kwon, Won, et al., 2010). The first authors have shown a molecule with a backbone of (1 → 6)-linked D-mannopyranosyl and (1 → 6)-linked D-glucopyranosyl residues, with D-Man*p* branches at O-4. The latter authors isolated a glucogalactomannan with a backbone of (1 → 2)-linked D-mannopyranosyl and (1 → 6)-linked D-mannopyranosyl residues, which occasionally branches at O-6.

Yu et al. (2007) have also isolated a glucogalactomannan from the fruit bodies of *C. militaris*, although its structure was quite different. The backbone of this heteropolysaccharide was composed of (1 → 6)-linked α-D-mannopyranosyl residues, being occasionally substituted at O-3 by α-D-glucopyranosyl and β-D-galactopyranosyl residues. An acid water-soluble polysaccharide, isolated from *C. sinensis* mycelium, had also showed mannose, glucose, and galactose at a ratio of 3.5:1:1.5 (Chen et al., 2010). This polymer promoted phagocytosis of RAW264.7 cells and stimulated NO production (Chen et al., 2010). The fungus of genus *Cordyceps* have been appreciated because of its therapeutic benefits, therefore, the study of their polysaccharide structures is necessary for providing new insights about mechanism of action of such molecules.

In contrast with the above mentioned authors, the backbone of the glucogalactomannan isolated from *C. militaris*, in the present study, was composed of (1 → 2)-linked α-D-mannopyranosyl residues instead of (1 → 6)-linkages. The branches occurred at O-6, mainly by (1 → 6)-linked-α-D-Man*p* or (1 → 2)-linked-β-D-Gal*f* fragments, that can be terminated with non-reducing end units of α-D-Man*p*, α-D-Gal*p*, α-D-Gal*f* or β-D-Gal*f*.

The galactomannan of the Ascomycete fungus *Sporonthrix schenckii* consists of a similar backbone of a core of 2-*O*- and 2,6-di-*O*-substituted α-D-mannopyranosyl units, with three or four consecutive (1 → 2) linkages occurring between them (Barreto-Bergter & Gorin, 1983).

Considering the complexity of analyzing these heteropolysaccharides, and that they may present a variety of main chains and side chains, the fraction R100-PD was submitted to a partial hydrolysis, with TFA, pH 2.0, at 100 °C, for 12 h. The resulting polymeric product was recovered by EtOH precipitation, as described in item

2.8, giving rise to the PH fraction. Its monosaccharide composition showed a reduction of the galactose and glucose amounts (Table 1), which was expected considering that the galactofuranose residues are more liable to mild hydrolysis than the pyranosyl monosaccharides. This reduction was confirmed by the ^{13}C-NMR experiment (Fig. 3) that presented higher resolution signals in the anomeric region as well as the signals relative to the (1 → 2) and (1 → 6) linkages, at δ 78.1; 78.6; and 65.7 ppm, respectively. No signals of α- or β-D-galactofuranose were observed in the PH spectrum, confirming its complete removal from the structure, showing that these residues were localized at the branching chains. The methylation analysis of PH confirmed these results, by the absence of the derivatives 2,3,5,6-Me$_4$-Gal*f* and 3,5,6-Me$_3$-Gal*f*. The increase observed in the amounts of 3,4,6-Me$_3$-Man*p* (42.7%) and 2,3,4-Me$_3$-Man*p* (30.7%) strongly indicates that this heteropolymer consists of a backbone of (1 → 2) linkages, and branches of (1 → 6) linkages. Considering the diversity of structures presented in this article, other possibilities of side-chains formations are not discarded.

The galactomannans are usually found in Lichens and pathogenic Ascomycetes (Ahrazem et al., 2006; Barreto-Bergter & Gorin, 1983; Cordeiro et al., 2005). These heteropolysaccharides, which contain high amounts of mannose and galactofuranose, were not observed for Basidiomycetes. The medicinal mushroom *C. militaris* is an entomopathogenic fungus that also belongs to the phylum Ascomycetes, and the present study showed that the chemical structure of its glucogalactomannan is in accordance with the other members of this taxonomic group.

4. Conclusions

The present study has shown the isolation and purification of a heteropolysaccharide from the alkaline extract of the fungus *C. militaris*. The spectroscopy and spectrometry analyses suggested that this polymer is a glucogalactomannan, with a backbone of (1 → 2)-linked-α-D-Man*p*, that can be substituted by (1 → 6)-linked-α-D-Man*p* or (1 → 2)-linked-β-D-Gal*f* fragments, terminating with α-D-Man*p*, α-D-Gal*p*, α-D-Gal*f* or β-D-Gal*f* units. The NMR data have also indicated side chains of α-D-Man*p*-(1 → 2)-α-D-Man*p*-(1 → 6)-β-D-Glc*p*-(1 → . The β-D-Glc*p* of this trisaccharide may be also substituted at O-4 by non-reducing end units as α-D-Gal*p*, α-D-Gal*f* or β-D-Gal*f*. The similarity of the glucogalactomannan structure with other heteropolysaccharides from Ascomycetes fungi just shows their taxonomic proximity.

Acknowledgements

The authors would like to thank Dr. J. M. Sung of Kangwon National University (Chuncheon, Korea), for the fungal material, and the Brazilian funding agencies Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), and the Fundação Araucária.

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