



Fucomannogalactan and glucan from mushroom *Amanita muscaria*: Structure and inflammatory pain inhibition



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ARTICLE INFO

Article history:

Received 30 March 2013

Received in revised form 25 June 2013

Accepted 27 June 2013

Available online 1 July 2013

Keywords:

Amanita muscaria

Fucomannogalactan

(1 → 3), (1 → 6) β-D-Glucan

Anti-inflammatory

Pain

ABSTRACT

A fucomannogalactan (FMG-Am) and a (1 → 3), (1 → 6)-linked β-D-glucan (βGLC-Am) were isolated from *Amanita muscaria* fruiting bodies. These compounds' structures were determined using mono- and bi-dimensional NMR spectroscopy, methylation analysis, and controlled Smith degradation. FMG-Am was shown to be a heterogalactan formed by a (1 → 6)-linked α-D-galactopyranosyl main chain partially substituted at O-2 mainly by α-L-fucopyranose and a minor proportion of β-D-mannopyranose non-reducing end units. βGLC-Am was identified as a (1 → 3)-linked β-D-glucan partially substituted at O-6 by mono- and a few oligosaccharide side chains, which was confirmed after controlled Smith degradation. Both the homo- and heteropolysaccharide were evaluated for their anti-inflammatory and antinociceptive potential, and they produced potent inhibition of inflammatory pain, specifically, $91 \pm 8\%$ (30 mg kg^{-1}) and $88 \pm 7\%$ (10 mg kg^{-1}), respectively.

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1. Introduction

Amanita muscaria (L.:Fr.) Person, commonly known as the fly agaric mushroom, is a native species throughout the temperate and boreal regions of the northern hemisphere. However, because this mushroom grows only in symbiosis with birch and/or pine (*Pinus* spp.) trees, it has been unintentionally introduced to many countries in the southern hemisphere.

Known as a toxic mushroom, *A. muscaria* contains muscarine, muscinol and ibotenic acid, in addition to muscazone, which are responsible for its psychoactive effects. Although the toxins in *A. muscaria* are water soluble, its consumption as a food has never been widespread. Nevertheless, the consumption of detoxified *A. muscaria* has been practiced in some localities in Europe and North America as sauce for steak (Coville, 1898) and in parts of Japan, such as in the Hokkaido area (Kiho, Katsuragawa, Nagai, & Ukai, 1992). The assumed toxic characteristics of *A. muscaria* may be derived from investigations focused on the psychoactive compounds present in its fruiting bodies.

Although it is well-known that many medicinal and therapeutic properties are attributed to polysaccharides derived from mushrooms, little is known regarding *A. muscaria* polymers.

Among polysaccharides, glucans are the most abundant and widely distributed carbohydrates in the fungal cell wall, and their biological activities are thought to result from the presence of β-D-glucans, often of the (1 → 3) linkage type (Kulicke, Lettau, & Heiko, 1997; Willment, Gordon, & Brown, 2001). Such polymers are considered the most interesting functional components in mushrooms (Manzi & Pizzoferrato, 2000). Consequently, there is great interest in these molecules, especially in their roles as biological response modifiers (Gonzaga, Ricardo, Heatly, & Soares, 2005; Lavi, Friesem, Geresh, Hadar, & Schwartz, 2006; Smith, Rowan, & Sullivan, 2002).

Conversely, heterogalactans, especially those that contains fucose, have been reported in basidiomycetes and exhibit enhanced biological activities (Cho, Koshino, Yu, & Yoo, 1998; Lu, Cheng, Lin, & Chang, 2010; Mukumoto & Yamaguchi, 1977). Most of these heterogalactans obtained from mushrooms, such as *Agaricus brasiliensis* and *Agaricus bisporus* var. *hortensis* (Komura et al., 2010), *A. bisporus* and *Lactarius rufus* (Ruthes, Rattmann, Carbonero, Gorin, & Iacomini, 2012; Ruthes et al., 2013a), *Flammulina velutipes* (Mukumoto & Yamaguchi, 1977; Smiderle et al., 2006), *Fomitella fraxinea* (Cho et al., 1998), *Fomitopsis pinicola* (Usui,

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Hosokawa, Mizuno, Suzuki, & Megur, 1981), *Ganoderma applanatum* (Usui, Iwasaki, & Mizuno, 1981), *Laetiporus sulphureus* (Alquini et al., 2004) and *Lentinus edodes* (Carbonero et al., 2008; Shida, Haryu, & Matsuda, 1975), have a backbone of (1 → 6)-linked- α -D-galactopyranosyl residues, substituted at O-2 only by L-Fucp or by L-Fucp in addition to D-Manp, D-Galp single units, or 3-O- α -D-mannopyranosyl- α -L-fucopyranosyl side chains.

Several physical and chemical properties, such as monosaccharide composition, molecular weight, degree of branching, glycosidic linkages and substituents, may influence the biological activities of polysaccharides (Bohn & BeMiller, 1995; Vetvicka & Yvin, 2004). Structural characterization of polysaccharides has become important for comparing chemical structure with biological properties.

In our recent studies, heterogalactans and branched (1 → 3), (1 → 6)-linked β -D-glucans were purified and structurally characterized from different mushroom species, and their antinociceptive and anti-inflammatory activities were evaluated and presented potent inhibition of inflammatory pain (Ruthes et al., 2012, 2013a, 2013b).

Consequently, the isolation, structural characterization and evaluation of the antinociceptive and anti-inflammatory potential of (1 → 3), (1 → 6)-linked β -D-glucan and fucomannogalactan from *A. muscaria* fruiting bodies described in this study may contribute to a structure–activity relationship, as they have differences in solubility, degrees of branching, molecular weight and substituents compared to similar polysaccharides that were previously described.

2. Materials and methods

2.1. Biological material

Amanita muscaria (L.:Fr.) Person fruiting bodies were collected in middle of May 2005 from the soil of a *Pinus* sp. reforestation project located in Mafra, Santa Catarina state, Brazil at latitude: 26°13'S, longitude: 49°50'W and an altitude of 826 m above sea level. They were identified by the mycologist André A. R. de Meijer, cleaned, vacuum dried, and subsequently ground to a powder.

2.2. Extraction and purification of polysaccharides

Extraction of crude polysaccharides obtained from *A. muscaria* (*Am*) and their purification was carried out as described in the flowchart (Fig. 1).

Dried milled powder from *A. muscaria* fruiting bodies (30.0 g) was submitted to successive cold (4 °C) and hot (~98 °C) aqueous and alkaline 2% KOH extraction, the latter under reflux at 100 °C for 6 h (6 ×, 1 L) each. The extracted polysaccharides were recovered from the aqueous extracts by the addition of excess EtOH or after dialysis from HOAc-neutralized alkaline extracts, resulting in fractions CW (cold water), HW (hot water) and K2 (alkaline 2%), respectively. The crude fractions obtained from hot aqueous (HW) and alkaline (K2) extractions were submitted to a freeze–thawing process (Gorin & Iacomini, 1984), furnishing cold water-soluble (SHW and SK2) and -insoluble polysaccharide fractions, which were separated by centrifugation (8000 rpm, 20 min, 5 °C). The water-soluble fractions were treated with Fehling solution (Jones & Stoodley, 1965), and the soluble fractions (FSHW and FSK2) were isolated from the insoluble Cu²⁺ complexes (FPHW and FPK2) by centrifugation under the same conditions as described above. The respective fractions were each neutralized with HOAc, dialyzed against tap water and deionized with mixed ion exchange resins and later freeze dried.

FPHW fraction was further purified by closed dialysis through a membrane with a 100 kDa M_w cut-off (Spectra/Por® Cellulose

Ester), giving rise to a retained (R-FPHW) and an eluted (E-FPHW) material (Fig. 1).

2.3. Monosaccharide composition

Each polysaccharide fraction (1 mg) was hydrolyzed with 2 M TFA at 100 °C for 8 h, followed by evaporation. The resulting residue was reduced with NaBH₄ (1 mg) and acetylated with Ac₂O–pyridine (1:1, v/v; 200 μ L) at 100 °C for 30 min following the method of Sasaki et al. (2008). The resulting alditol acetates were analyzed by gas chromatography–mass spectrometry (GC–MS) using a Varian model 3300 gas chromatograph linked to a Finnigan Ion-Trap, Model 810-R12 mass spectrometer. A DB-225 capillary column (30 m × 0.25 mm i.d.) held at 50 °C during injection and later programmed to 220 °C (constant temperature) at 40 °C min^{−1} was used for qualitative and quantitative analysis of alditol acetates. The alditol acetates were identified by their typical retention times and electron impact profiles.

2.4. Determination of homogeneity and polymer molecular weight (M_w)

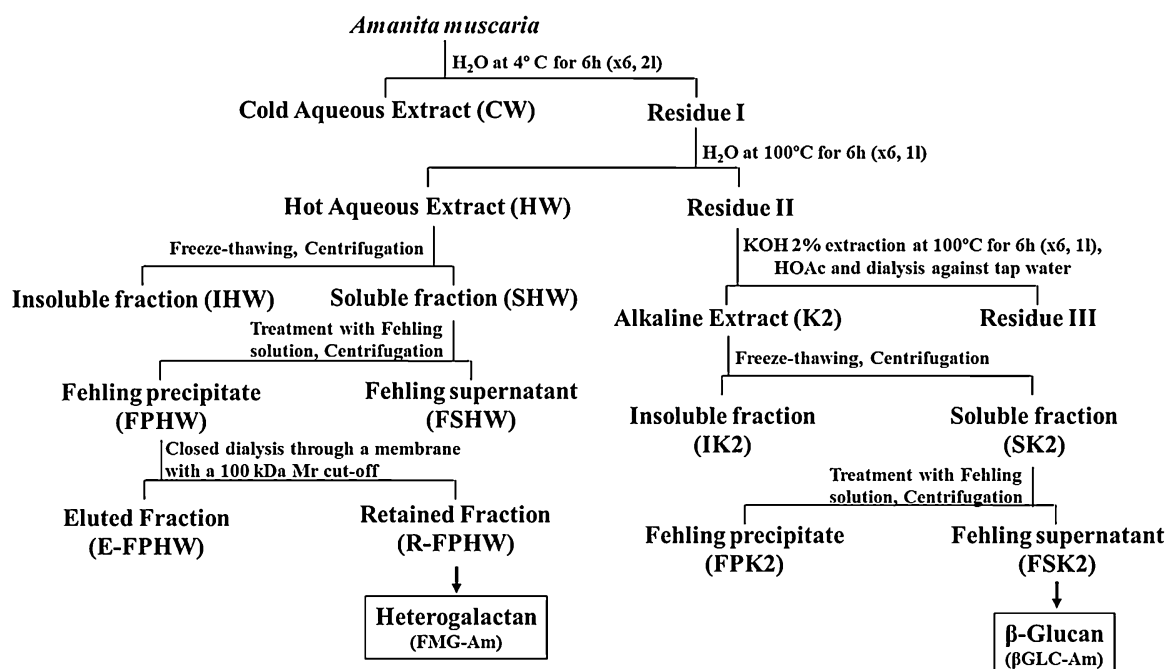
The homogeneity and molecular weight (M_w) of water-soluble purified polysaccharide fractions FMG and β GLC were determined by high-performance steric exclusion chromatography (HPSEC) using a refractive index (RI) detector. The eluent was 0.1 M NaNO₃ containing 0.5 g L^{−1} NaN₃. The solutions were filtered through a membrane with pores of 0.22- μ m diameter (Millipore). The specific refractive index increment (dn/dc) was determined using a Waters 2410 detector. The samples were dissolved in the eluent in five increasing concentrations ranging from 0.2 to 1.0 mg mL^{−1} to determine the slope of the increment.

2.5. Methylation analysis

Per-O-methylation of purified FMG-*Am* and β GLC-*Am* fractions (10 mg each) was carried out using NaOH–Me₂SO–MeI, a method modified from Ciucanu and Kerek (1984) as described by Ruthes et al. (2010). The per-O-methylated derivatives were hydrolyzed with 45% formic acid (HCO₂H, 1 mL) for 15 h at 100 °C (Carbonero et al., 2012), followed by NaBD₄ reduction and acetylation as above (Section 2.3) to give a mixture of partially O-methylated alditol acetates that was analyzed by GC–MS using a Varian model 4000 gas chromatograph equipped with DB-225 capillary columns. The injector temperature was maintained at 210 °C, with the oven increasing from 50 °C (hold 2 min) to 90 °C (20 °C min^{−1}, then held for 1 min), 180 °C (5 °C min^{−1}, then held for 2 min) and to 210 °C (3 °C min^{−1} and held for 5 min). Helium was used as the carrier gas at a flow rate of 1.0 mL min^{−1}. Partially O-methylated alditol acetates were identified from m/z by comparing their positive ions with standards, with the results being expressed as a relative percentage of each component (Sasaki, Gorin, Souza, Czelusniak, & Iacomini, 2005).

2.6. Controlled Smith degradation of β -D-glucan

The purified glucan (β GLC-*Am*; 100 mg) was submitted to oxidation with 0.05 M aqueous NaIO₄ (15 mL) at room temperature for 72 h in the dark. Ethylene glycol was added to stop the reaction, the solution was dialyzed, and the resulting polyaldehydes were reduced with NaBH₄ for 24 h, neutralized with HOAc, dialyzed, and concentrated (Goldstein, Hay, Lewis, & Smith, 2005). The residue was partially hydrolyzed with TFA pH 2.0 (30 min at 100 °C) (Gorin, Horitsu, & Spencer, 1965) followed by dialysis against tap water using a membrane with a size exclusion of 2 kDa, and the solution containing the retained material was freeze-dried. An aliquot



Fraction SHW gave a heterogeneous HPSEC elution profile; therefore, it was purified by treatment with Fehling solution followed by closed dialysis of fraction FPHW (1.02 g, 3.4% yield) through a 100 kDa M_w cut-off membrane (Fig. 1). The retained fraction (R-FPHW; 0.96 g, 3.2% yield) was homogeneous by HPSEC, and had a M_w 25.5×10^3 g mol⁻¹ (dn/dc 0.163 mL g⁻¹) (Fig. 2A). This fraction contained fucose (22%), mannose (9%) and galactose (69%)

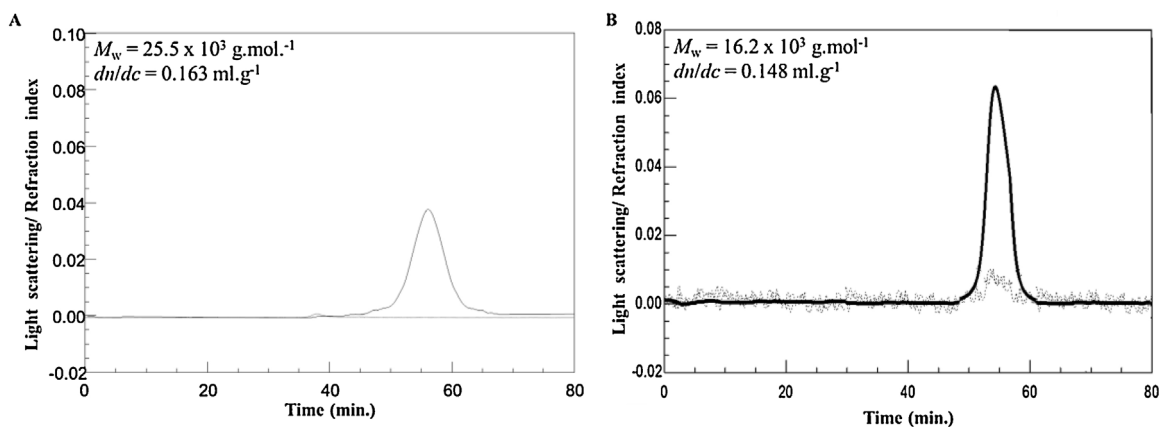


Fig. 2. Elution profiles of fractions FMG-Am (A) and β GLC-Am (B) determined by HPSEC using light scattering (—) and refractive index detectors (---).

as monosaccharide components, suggesting the presence of a heterogalactan, and it was named FMG-Am.

Analysis by GC–MS of the partially *O*-methylated alditol acetates of FMG-Am suggested the existence of a highly branched structure consistent with a fucomannogalactan due to the presence of partially *O*-methylated derivatives 2,3,4-Me₃-Fuc (21.6%), 2,3,4,6-Me₄-Man (8.9%), 2,3,4-Me₃-Gal (37.6%), and 3,4-Me₂-Gal (31.9%) (Table 1).

NMR analysis [¹³C- (Fig. 3A), DEPT (Fig. 3A') and HSQC (Fig. 3B)] contributed to the elucidation of the fucomannogalactan (FMG-Am) structure because the coupling of protons of its units enable assignments of their respective carbons using HSQC analysis (Fig. 3B). The HSQC spectrum of FMG-Am contained H-1 signals corresponding to non-reducing end groups of D-Manp (δ 4.83) and L-Fucp (δ 5.11), as well as units of 6-*O*- (δ 5.02) and 2,6-di-*O*-substituted D-Galp (δ 5.17, 5.16, and 5.08) (Table 2). The ¹³C NMR spectrum (Fig. 3A) of FMG-Am shows an overlapping of signals relative to D-Manp and L-Fucp non-reducing end units; the HSQC spectrum (Fig. 3B') had signals (C-1/H-1) at δ 104.0/4.83 and 103.9/5.11 corresponding to D-Manp and L-Fucp units, respectively (Table 2).

As for the C-1 signals from the D-Manp and L-Fucp units, those from 2,6-di-*O*- (δ 101.0/5.16, 100.7/5.17 and 100.7/5.08), and 6-*O*-substituted D-Galp residues (δ 100.3/5.02) could only be attributed after HSQC analysis (Fig. 3B'; Table 2) because the ¹³C NMR spectrum of FMG-Am presents a superposition of signals at the anomeric region at δ 100.6. The glycosidic configurations were confirmed by values of the coupling constants $J_{C-1,H-1}$ found in ¹H/¹³C coupled HMQC spectrum (Perlin & Casu, 1969). The non-reducing ends of

D-Manp have a β -configuration consistent with a value of 162.8 Hz. The residues of D-Galp and L-Fucp had an α -configuration in agreement with a 172.3 Hz coupling constant.

The 6-*O*-substituted signals of D-Galp units were present at δ 69.2, 69.4, 69.7, 69.8, and 70.1 in the ¹³C NMR (Fig. 3A) and HSQC spectra (Fig. 3B), which were confirmed from the inverted signals in its DEPT spectrum (Fig. 3A').

The signals at δ 72.8/4.21, 75.4/3.67, 69.5/3.63, 78.6/3.41, and 63.5/3.96; 3.78 arose from C-2/H-2 to C-6/H-6 of the D-Manp units, respectively, while those at δ 72.3/3.82, 71.3/3.84, 74.2/3.88, 72.2/4.16, and 18.1/1.27 were from similar C-2/H-2 to C-6/H-6 correlations of L-Fucp residues (Table 2) (Carbonero et al., 2008).

According to the literature, the C-2 of D-Galp units *O*-substituted by D-Manp could be assigned at δ 79.4. These D-Galp units also had signals of C-1/H-1 at δ 101.0/5.16 and 100.7/5.17 (Carbonero et al., 2008). The C-2 signal of D-Galp units *O*-substituted by L-Fucp appeared at δ 80.3 and corresponds to D-Galp units with C-1/H-1 at δ 100.7/5.08 (Carbonero et al., 2008).

The results of the monosaccharide composition analyses, methylation data, and NMR analysis of FMG-Am show it to be a fucomannogalactan containing a (1 $\rightarrow$ 6)-linked, α -D-galactopyranosyl main chain that is partially substituted at *O*-2 mainly by α -L-fucopyranose and in a minor proportion by β -D-mannopyranose as non-reducing end units.

Polysaccharides resembling FMG-Am have been previously identified in extracts of fruiting bodies of *L. edodes* (Carbonero et al., 2008; Shida et al., 1975), where both L-fucose and D-mannose are present exclusively as terminal residues in the heterogalactan, as

Table 1
Partially *O*-methylated alditol acetates formed on methylation analysis of the fucomannogalactan (FMG-Am) and β -D-glucan (β GLC-Am) obtained from *A. muscaria* fruiting bodies: linkage types.

Partially <i>O</i> -methylated alditol acetates ^a	Rt ^b	% Area of fragments ^c			Linkage type
		FMG ^d	β GLC ^e	SM- β GLC ^f	
2,3,4-Me ₃ -Fuc	6.507	21.6	–	–	Fucp-(1 $\rightarrow$
2,3,4,6-Me ₄ -Man	7.726	8.9	–	–	Manp-(1 $\rightarrow$
2,3,4,6-Me ₄ -Glc	7.985	–	29.9	2.5	Glc p-(1 $\rightarrow$
2,4,6-Me ₃ -Glc	10.039	–	40.3	95.3	3 $\rightarrow$)-Glc p-(1 $\rightarrow$
2,3,4-Me ₃ -Gal	10.737	37.6	–	–	6 $\rightarrow$)-Galp-(1 $\rightarrow$
3,4-Me ₂ -Gal	12.156	31.9	–	–	2,6 $\rightarrow$)-Galp-(1 $\rightarrow$
2,4-Me ₂ -Glc	14.627	–	29.8	2.2	3,6 $\rightarrow$)-Glc p-(1 $\rightarrow$

^a GC–MS analysis on a DB-225 capillary column.

^b Retention time (min).

^c Based on derived *O*-methylalditol acetates.

^d FMG-Am: fucomannogalactan isolated from *A. muscaria*.

^e β GLC-Am: – (1 $\rightarrow$ 3), (1 $\rightarrow$ 6)-linked β -D-glucan isolated from *A. muscaria*.

^f SM- β GLC – β -D-glucan product after controlled Smith degradation.

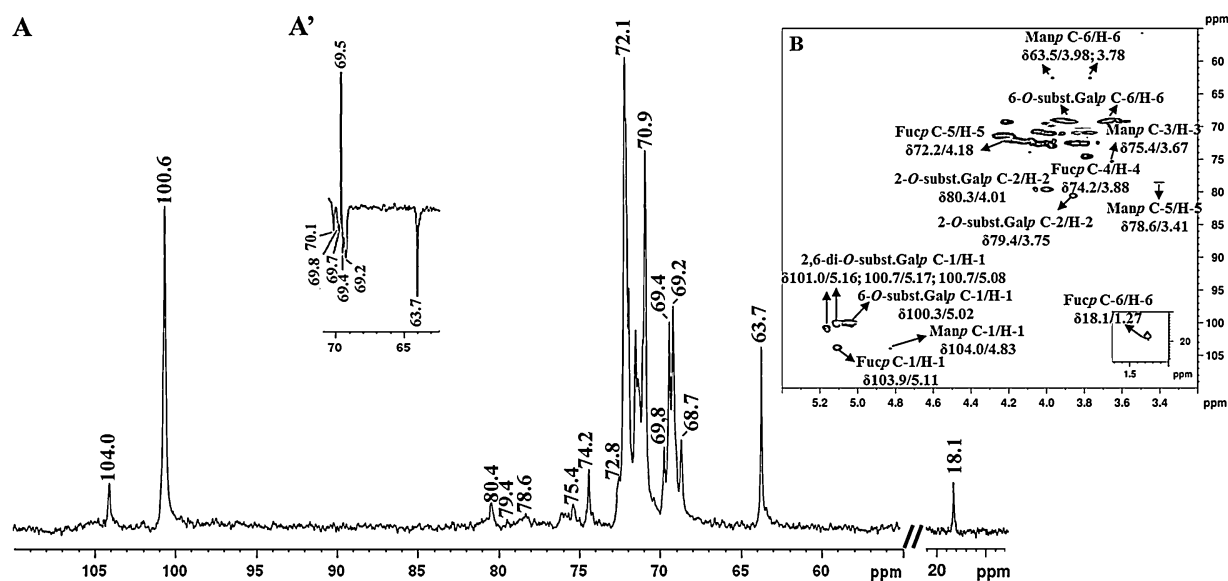


Fig. 3. ¹³C NMR (A), with inserts of DEPT-CH₂ inversion (A') and ¹H (obs.)/¹³C HMQC (B) spectra of fucomannogalactan; FMG-Am was analyzed in D₂O at 70 °C (chemical shifts are expressed in δ ppm).

described in this study for *A. muscaria*. The difference observed is related to D-Manp and L-Fucp content as non-reducing end units; in FMG from *A. muscaria*, L-Fucp (21%) appears in greater amount than D-Manp (2%), and in *L. edodes* fucomannogalactan, the inverse is observed (D-Manp – 21% and L-Fucp – 11%) (Carbonero et al., 2008).

There have been several other reports dealing with the isolation and characterization of heterogalactans of basidiomycetes. Most of these compounds have a common structure consisting of a backbone of (1 → 6)-linked, α-D-galactopyranose residues, some of which are substituted at C-2 either by L-fucopyranose or 3-O-mannopyranosyl-L-fucopyranosyl residues, such as those described for *Armillaria mellea* (Bouveng, Fraser, & Lindberg, 1967; Fraser & Lindberg, 1967), *F. fraxinea* (Cho et al., 1998; Cho, Yun, Yoo, & Koshino, 2011), *G. applanatum* (Usui et al., 1981b), *F. velutipes* (Mukumoto & Yamaguchi, 1977; Smiderle et al., 2006), *L. sulphureus* (Alquini et al., 2004), *Polyporus fomentarius* (Björnlund & Lindberg, 1969), *Polyporus giganteus* (Bhavanandan, Bouveng, & Lindberg, 1994), *Polyporus pinicola* (Fraser, Karacsony, & Lindberg,

1967), and *Polyporus squamosus* (Björndal & Wagstrom, 1969). Heterogalactans lacking D-Mannopyranose as end-units, known as fucogalactans, have also been described for mushrooms in *A. bisporus* (Ruthes et al., 2012, 2013a), *A. brasiliensis* and *A. bisporus* var. *hortensis* (Komura et al., 2010), *Coprinus commatus* (Fan et al., 2006), *Hericium erinaceus* (Zhang et al., 2006), and *L. rufus* (Ruthes et al., 2012).

In addition to presenting well-known chemical structures, heterogalactans are also recognized for their relevant biological activities (Carbonero et al., 2008; Fan et al., 2006; Komura et al., 2010; Ruthes et al., 2012, 2013a).

3.2. Structural characterization of (1 → 3), (1 → 6) β-D-glucan

For β-D-glucan purification, extract K2 was primarily submitted to the freeze–thawing procedure (Corin & Iacomini, 1984), resulting in the respective cold water-soluble fraction SK2 (750 mg, 2.5%), which yielded a heterogeneous HPSEC elution profile. Therefore, the fraction was purified by treatment with Fehling solution. FSK2

Table 2

¹H and ¹³C NMR chemical shifts [expressed as δ (ppm)] of fractions FMG-Am, βGLC-Am and SM-βGLC from *A. muscaria*^a.

Units			1	2	3	4	5	6	
								a	b
FMG-Am	α-L-Fucp-(1→	¹³ C	103.9	72.3	71.3	74.2	72.2	18.1	–
		¹ H	5.11	3.82	3.84	3.88	4.16	1.27	–
	β-D-Manp-(1→	¹³ C	104.0	72.8	75.4	69.5	78.6	63.5	63.5
		¹ H	4.83	4.21	3.67	3.63	3.41	3.96	3.78
	2,6→)-α-D-Galp-(1→	¹³ C	100.7/101.0	80.3	71.1	71.2	71.7	69.7	69.7
		¹ H	5.17;5.08/5.16	3.84	4.08	4.09	4.21	3.70	4.01
βGLC-Am	6→)-α-D-Galp-(1→	¹³ C	100.3	71.1	72.4	72.4	71.7	69.4	69.4
		¹ H	5.02	3.84	3.89	4.03	4.21	3.72	3.93
	β-D-Glcp-(1→	¹³ C	103.0	73.4	76.0	70.3	75.0	60.9	60.9
		¹ H	4.30	3.13	3.29	3.23	3.41	3.70	3.53
	3→)-β-D-Glcp-(1→	¹³ C	102.9	72.8	85.5/85.0	68.3	75.7	60.9	60.9
		¹ H	4.51	3.32	3.53	3.31	3.41	3.70	3.53
SM-βGLC	3,6→)-β-D-Glcp-(1→	¹³ C	102.6	72.8	84.8/84.7	68.3	75.7	68.8	68.8
		¹ H	4.51	3.32	3.53	3.31	3.41	3.98	3.98
	3→)-β-D-Glcp-(1→	¹³ C	102.9	72.8	86.1	68.4	76.3	60.9	60.9
		¹ H	4.43	3.23	3.40	3.18	3.18	3.69	3.40

^aAssignments are based on ¹³C, ¹H, DEPT, COSY and HMQC analysis.

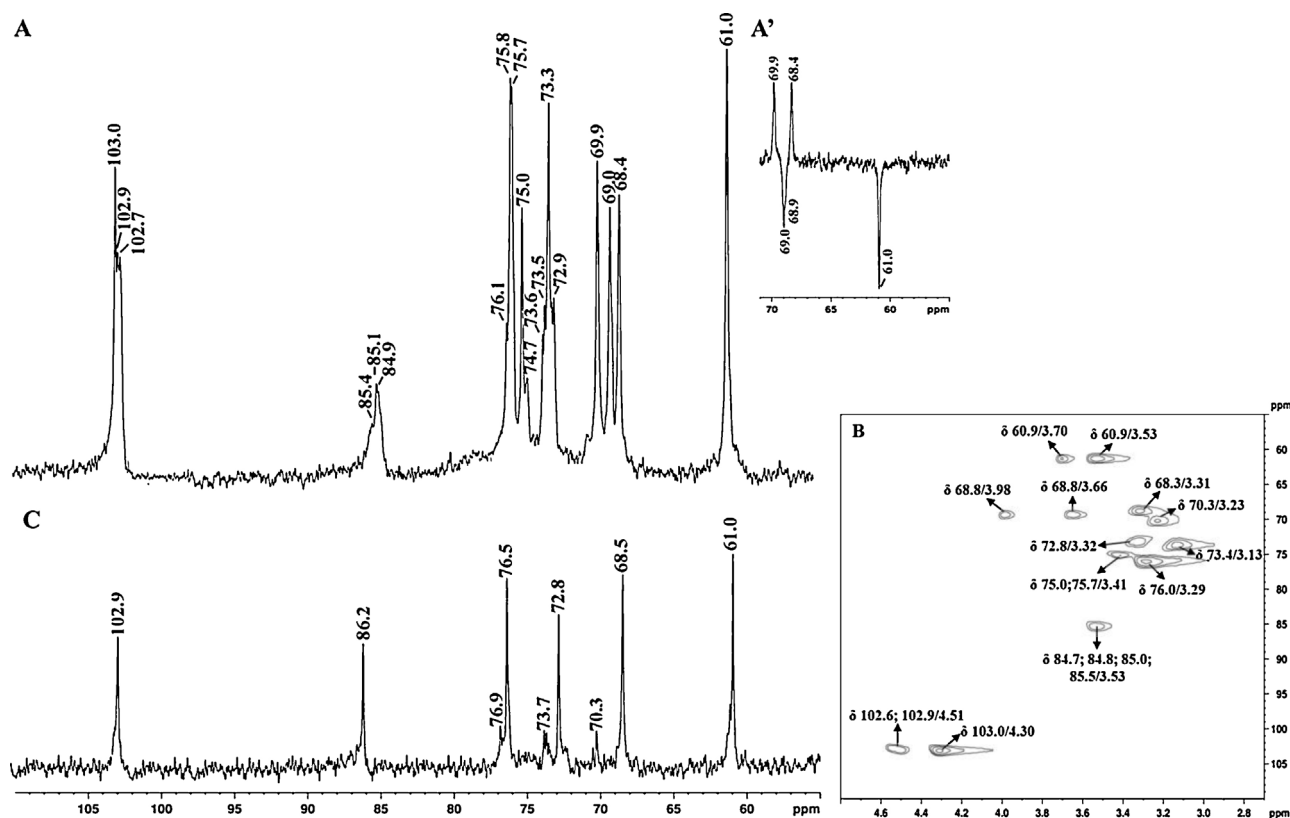


Fig. 4. ^{13}C NMR spectrum of β -D-glucan (A), with insert of DEPT- CH_2 inversion (A'); ^1H (obs.)/ ^{13}C HMQC spectrum of β -GLC-Am (B), and ^{13}C NMR spectrum of its Smith-degraded product (C). Analysis were done in D_2O (A and C) and $\text{Me}_2\text{SO}-d_6$ (B) at 70 °C (chemical shifts are expressed in δ ppm).

(366 mg, 1.2%) proved to be homogeneous on HPSEC with a M_w $16.2 \times 10^3 \text{ g mol}^{-1}$ (dn/dc 0.148 mL g^{-1}) (Fig. 2B).

Fraction FSK2 contained only glucose as its monosaccharide component (GC-MS), and it was named β -GLC-Am. Analysis by GC-MS of its partially O-methylated alditol acetates suggested the existence of a branched (1 $\rightarrow$ 3), (1 $\rightarrow$ 6)-linked β -D-glucan due to the presence of partially O-methylated derivatives 2,3,4,6-Me₄-Glc, 2,4,6-Me₃-Glc, and 2,4-Me₂-Glc, determined in a molar ratio of $\sim 1:1.4:1$ (Table 1).

Fraction β -GLC-Am was examined using NMR spectroscopy, and signals were assigned using 1D (^1H , ^{13}C , and DEPT) and 2D NMR spectra (^1H (obs.)/ ^{13}C HMQC and COSY). All of the signals were compared to literature values for similar polysaccharides (Carbonero et al., 2006; Chauveau, Talaga, Wieruszski, Strecker, & Chavant, 1996; Ruthes et al., 2013b; Santos-Neves et al., 2008; Smiderle et al., 2006, 2008; Tabata, Ito, & Kojima, 1981; Yoshioka, Tabeta, Saito, Uehara, & Fukuoaka, 1985).

^{13}C NMR (Fig. 4A), and ^1H (obs.)/ ^{13}C HMQC spectra (Fig. 4B) of β -D-glucan contained three distinct signals in the anomeric region at δ 103.0/4.30, 102.9/4.51 and 102.7/4.56 (Table 2), corresponding to the non-reducing end, and 3-O- and 3,6-di-O-substituted residues, respectively. The β -configuration was confirmed by low-frequency of H-1 (δ 4.56, 4.51, 4.30 and 4.21) and high-frequency C-1 signals (δ 103.1, 102.9 and 102.7) (Fig. 4A and B) (Hall & Johnson, 1969). The glycosidic linkages of the glucan were shown by the presence of 3-O-substituted signals at δ 85.4, 85.1 and 84.9 for β -GLC (Fig. 4A). The O-6 substitution was confirmed from the respective reversed peak in the DEPT spectrum (Fig. 4A'), and it also appeared as doublet in HMQC at δ 68.8; 3.98/3.66 (Fig. 4B).

The backbone structure of the isolated glucan was characterized by controlled Smith degradation, and its remaining product (SM- β -GLC) was analyzed by ^{13}C NMR (Fig. 4C) spectroscopy and

^1H (obs.)/ ^{13}C HMQC (data not shown). It can be assumed that the β -GLC-Am fraction has a linear main-chain formed by (1 $\rightarrow$ 3)-linked β -D-glucan due to the presence of typical C/H signals shown in Table 2, at δ 102.9/4.43 (C-1/H-1); 86.1/3.40 (C-3/H-3); 76.3/3.18 (C-5/H-5); 72.8/3.23 (C-2/H-2); 68.4/3.18 (C-4/H-4), and 60.9/3.69; 3.40 (C-6/H-6a;b) (Gorin, 1981). In the degraded fraction of the soluble glucan (SM- β -GLC), low-intensity signals from O-6 substitution were still present (Fig. 4C), indicating that this glucan is mostly substituted at O-6 by single-units of β -D-Glcp and a minor proportion by side chains of 3-O-substituted β -D-glucopyranosyl oligosaccharides.

The product of controlled Smith degradation was methylated, and the alditol GC-MS analysis confirms the NMR data. The SM- β -GLC fraction presented not only substitutions by non-reducing end units but also substitution by (1 $\rightarrow$ 3)-linked β -D-Glcp side chains (Table 1) (Carbonero et al., 2006; Chauveau et al., 1996; Ruthes et al., 2013b; Santos-Neves et al., 2008; Smiderle et al., 2008; Yoshioka et al., 1985).

In conclusion, the results of the monosaccharide composition, methylation data, NMR spectroscopic analysis, and controlled Smith degradation of the studied fraction β -GLC-Am indicated the presence of a β -D-glucan with a main-chain that is (1 $\rightarrow$ 3)-linked, partially substituted at O-6 mostly by non-reducing end units of β -D-Glcp as side-chains, and a minor proportion 3-O-substituted by β -D-glucopyranosyl oligosaccharides.

β -GLC-Am appears to be similar to the soluble glucan purified from *L. rufus* fruiting bodies (Ruthes et al., 2013b). The structure we described for *A. muscaria* is less branched than that described for *L. rufus* but more branched than the previous β -D-glucan described for *A. muscaria*, where branches were primarily single (1 $\rightarrow$ 6)-linked, two for every seven residues in the (1 $\rightarrow$ 3)-linked main chain (Kiho et al., 1992).

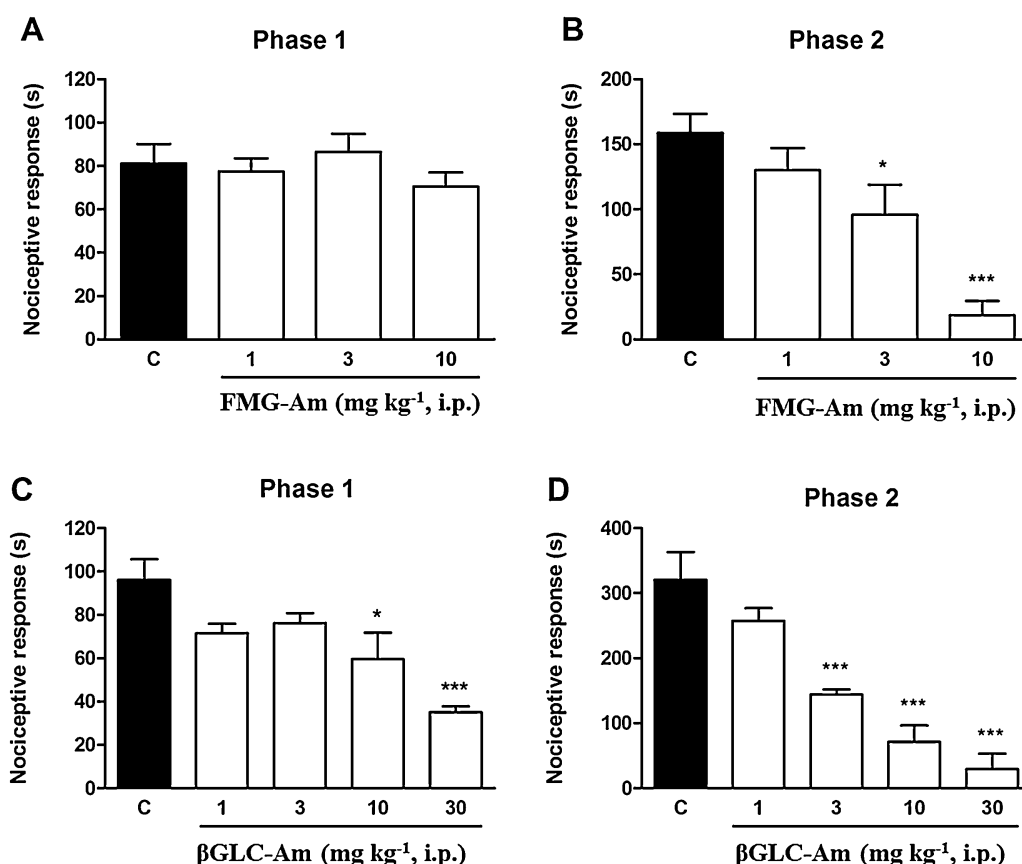


Fig. 5. Effects of intraperitoneally administered FMG-Am and β GLC-Am on formalin-induced nociception (early phase, panels A and C; and late phase, panels B and D) in mice. Each column represents the mean $4-6$ animals $\pm$ S.E.M. Control values (C) indicate the animals injected with saline or vehicle (saline plus 5% Tween 80) and the asterisks denote the significance levels when compared with the control group; * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ (One-way ANOVA followed by the Newman–Keuls test).

3.3. Effects of FMG-Am and β GLC-Am on nociception induced by intraplantar injection of formalin

Pain remains an important health problem; in certain patients, it could be controlled by better application of existing therapies, but other conditions lack effective treatments. Recent studies have been demonstrated that mushroom extracts and isolated polysaccharides from their fruiting bodies have presented anti-inflammatory and antinociceptive effects.

In this study, we evaluated the possible anti-inflammatory and antinociceptive effects of a heterogalactan (FMG-Am) and a β -D-glucan (β GLC-Am) isolated from *A. muscaria* in the formalin model, a widely used model of persistent pain that is a mainstay for the development of novel agents for the treatment of postoperative pain (Shields, Cavanaugh, Lee, Anderson, & Basbaum, 2010).

It is well-established that the nociception promoted by the injection of formalin consists of two phases: neurogenic pain (early phase), which is provoked by direct activation of nociceptors, and inflammatory pain (late phase) that is mediated by a combination of peripheral input and spinal cord sensitization, mainly due to release of pro-inflammatory mediators (Hunnskaar & Hole, 1987; Tjølsen, Berge, Hunnskaar, Rosland, & Hole, 1992).

Our results demonstrated that intraperitoneal administration of β GLC-Am reduced the neurogenic pain (early phase) with an ID_{50} value of 16.54 ($8.64-31.65$) mg kg^{-1} and inhibition of $64 \pm 3\%$ at dose of 30 mg kg^{-1} (Fig. 5A), while FMG-Am did not produce an inhibitory effect on the early phase at all tested doses (Fig. 5C). However, β GLC-Am and FMG-Am were more effective against the inflammatory pain (late phase) of formalin-induced nociception with ID_{50} values of 2.41 ($1.13-5.14$) and 3.41 ($2.10-5.52$) mg kg^{-1} ,

respectively, and inhibition of $91 \pm 8\%$ at dose of 30 mg kg^{-1} of β GLC-Am and $88 \pm 7\%$ at a dose of 10 mg kg^{-1} of FMG-Am (Figs. 5B and D).

Interestingly, it was demonstrated that a similar fucomannogalactan isolated from *L. edodes* also showed antinociceptive and anti-inflammatory properties using the model of abdominal constrictions induced by acetic acid (Carbonero et al., 2008), which, additionally, involves endogenous inflammatory mediators. This polymer (100 mg kg^{-1}) inhibited the nociceptive response, leukocyte infiltration and peritoneal capillary permeability by 97, 100 and 76%, respectively (Carbonero et al., 2008).

The effects on nociception induced by intraplantar injection of formalin of *A. muscaria* FMG-Am ($2.2 \times 10^4 \text{ g mol}^{-1}$) were compared with those observed for *A. bisporus* fucogalactan ($37.1 \times 10^4 \text{ g mol}^{-1}$) using the same model (Ruthes et al., 2013a). FMG-Am was capable of inhibiting the inflammatory pain at a lower dose (10 mg kg^{-1} ; $88 \pm 7\%$), while the fucogalactan presented similar inhibition ($82 \pm 9\%$) but at a higher dose (100 mg kg^{-1}) (Ruthes et al., 2013a). The potency of the inhibitory effect was also different when comparing both heterogalactans. FMG-Am proved to be more potent [ID_{50} 3.41 ($2.10-5.52$) mg kg^{-1}] than the fucogalactan from *A. bisporus* [ID_{50} 36.0 ($25.0-50.3$) mg kg^{-1}]. Both are heterogalactans with the same main structure, (1 $\rightarrow$ 6)-linked α -D-galactopyranosyl units, partially substituted at O-2 mainly by α -L-fucopyranose. However, while FMG-Am also presents O-2 substitution by D-Manp units, *A. bisporus* fucogalactan does not but presents 3-O-Me-D-Galp units at the main chain (Ruthes et al., 2013a). These differences in structures and molecular weight could be responsible for the differences observed, especially regarding the potency of inhibitory effects.

Regarding the β -D-glucan, the soluble (1 $\rightarrow$ 3), (1 $\rightarrow$ 6) β -D-glucan previously isolated from *L. rufus* inhibited the nociceptive response induced by formalin with an ID₅₀ value of 2.35 (1.48–3.75) mg kg⁻¹ for the late phase (Ruthes et al., 2013b). This polysaccharide exhibits a higher molecular weight (11.3 $\times$ 10⁴ g mol⁻¹) and degree of branching than the (1 $\rightarrow$ 3), (1 $\rightarrow$ 6) β -D-glucan (β GLC-Am) (1.6 $\times$ 10⁴ g mol⁻¹) isolated from *A. muscaria* at the same phase [ID₅₀ 2.41 (1.13–5.14) mg kg⁻¹]. However, their inhibitory effects observed in the late phase of the formalin test are not significantly different, and the potency based on their ID₅₀ values is similar. Conversely, the inhibition observed on neurogenic pain was more pronounced for β GLC-Am (64 $\pm$ 3%), when compared with *L. rufus* β -D-glucan (36 $\pm$ 8%) (Ruthes et al., 2013b).

When comparing the β GLC-Am inhibitory effect against inflammatory pain (91 $\pm$ 8%) with the almost linear product (86 $\pm$ 9%) produced after controlled Smith degradation of the soluble (1 $\rightarrow$ 3), (1 $\rightarrow$ 6)-linked β -D-glucan from *L. rufus* (Ruthes et al., 2013b), we observed that the degree of branching followed by the resultant difference in solubility have great importance for the antinociceptive effect of β -D-glucans in response to inflammatory pain.

Acknowledgements

The authors would like to thank the Brazilian funding agencies: CAPES (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior), CNPq (Conselho Nacional de Desenvolvimento Científico e Tecnológico), Fundação de Amparo à Pesquisa e Inovação do Estado de Santa Catarina (FAPESC) and Fundação Araucária for financial support, and the mycologist André A. R. de Meijer for mushroom identification. A. C. Ruthes is beneficiary of post-doctoral scholarship from CAPES-PNPD; C.H. Baggio is beneficiary of post-doctoral scholarship from CNPq.

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