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Lactarius rufus (1→3),(1→6)-β-D-glucans: Structure, antinociceptive and anti-inflammatory effects

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ABSTRACT

Medicinal health benefits uses of edible as well as non-edible mushrooms have been long recognized. The pharmacological potential of mushrooms, especially antitumor, immunostimulatory and anti-inflammatory activities has been documented. Wild ectomycorrhizal mushroom, *Lactarius rufus* had the anti-inflammatory and antinociceptive potential of their polysaccharides evaluated using the formalin model. Two structurally different (1→3),(1→6)-linked β-D-glucans were isolated from fruiting bodies. Soluble (FSHW) β-D-glucan 1–30 mg kg^{-1} produced potent inhibition of inflammatory pain caused by formalin when compared with the insoluble one (IHW), suggesting that solubility and/or branching degree could alter the activity of β-glucans. Their structures were determined using mono- and bi-dimensional NMR spectroscopy, methylation analysis, and controlled Smith degradation. They were β-D-glucans, with a main chain of (1→3)-linked Glc*p* residues, substituted at O-6 by single-unit Glc*p* side chains (IHW), on average to every fourth residue of the backbone, or by mono- and few oligosaccharide side chains for soluble β-glucan.

1. Introduction

Wild mushrooms have become more important in our diet for their nutritional (Breene, 1990; Crisan & Sands, 1978; Manzi, Gambelli, Marconi, Vivanti, & Pizzoferrato, 1999), organoleptic (Maga, 1981) and pharmacological (Bobek, Ginter, Jurcovicová, & Kuniak, 1991; Bobek, Ozdyn, & Kuniak, 1995; Bobek & Galbavy, 1999) characteristics. Some of them can form mycorrhiza and as they are almost the only organisms that can degrade cellulose and lignin, they are indispensable in ecosystems (Gussem, Vandenabeele, Verbeken, & Moens, 2005). Some ectomycorrhizal species, such as *Lactarius rufus*, were introduced into Brazil due to the expansion of the timber industry, with increased *Eucalyptus* and *Pinus* reforestation.

Regarding the mentioned species, *L. rufus* fruiting bodies had not previously been studied in relation to its content of polysaccharides. Overall, those isolated from mushroom fruiting bodies can be either water-soluble or/and -insoluble homo- and heteropolysaccharides with different main- and side-chains.

Homo- and hetero-β-glucans with β-(1→3), β-(1→4) and β-(1→6) glycosidic linkages are considered the most interesting functional components in mushrooms (Manzi & Pizzoferrato, 2000). Consequently there is great interest on these molecules especially because they can act as biological response modifiers (Gonzaga, Ricardo, Heatly, & Soares, 2005; Lavi, Friesem, Geresh, Hadar, & Schwartz, 2006; Smith, Rowan, & Sulliva, 2002), and rather than attack the harmful agent they boost the host defense mechanism, stimulating its intrinsic ability to resist disease-causing invaders (Kogan, 2000). β-Glucans are supposed to play a key role in some healthy properties attributed to mushrooms, such as enhancement of macrophage function and host resistance to many bacterial, viral, fungal and parasitic infections and activation of a non-specific immune stimulation (Cheung, 1998; Manzi & Pizzoferrato, 2000; Rajarathnam, Shashirekha, & Bano, 1998).

The protective action of β-glucans, described as non-specific immunomodulators, may usually involve many different pathways including T-cell stimulation, increased macrophage participation and release of different cytokines, stimulation of

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reticulo-endothelial system (RES), activation of natural killer (NK) cells, activation of the classical and alternative complement pathways, increased antibody production (Kogan, 2000; Mantovani et al., 2008).

Studies *in vitro* and *in vivo* reveal that the immunostimulating activity of β-glucans depends on their structure, molecular weight and degree of branching (Brown & Gordon, 2003; Mantovani et al., 2008; Miura et al., 2003).

Consequently, the isolation and structural characterization of (1→3),(1→6)-linked β-D-glucans from *L. rufus* fruiting bodies are now described. Since they have differences in solubility, and different degrees of branching, the structures were also evaluated for antinociceptive and anti-inflammatory potential in order to allow a structure activity relationship.

2. Materials and methods

2.1. Biological material

L. rufus (Scop.: Fr.) Fr. fruiting bodies were collected in the middle of May, 2005 from soil of a *Pinus* sp. reforestation project located in Mafra, State of Santa Catarina, Brazil at latitude: 26°13′S; longitude: 49°50′W and altitude of 826 m above sea level. They were identified by the mycologist André A. R. de Meijer. The samples, after cleaning and drying *in vacuo*, were ground to a fine powder.

2.2. Extraction and purification of β-glucans

Extraction of crude polysaccharides obtained from *L. rufus*, and their purification was carried out as described in flowchart Fig. 1A.

Dried, Wiley-milled powder from *L. rufus* fruiting bodies (42.2 g) was submitted to successive cold (4 °C) and hot (~98 °C) aqueous extraction, both for 6 h (×6, 2 L and 1 L, respectively), each. The extracted polysaccharides were recovered from aqueous extracts by addition to excess EtOH, giving fractions CW and HW, respectively. The crude fraction obtained from hot aqueous extraction (HW), was submitted to a freeze-thawing process (Gorin & Iacomini, 1984) furnishing cold water-soluble (SHW) and -insoluble polysaccharide fractions (IHW), which were separated by centrifugation (8000 rpm, 20 min, 5 °C). The water-soluble fraction was treated with Fehling solution and the soluble fraction (FSHW) was isolated of the insoluble Cu^{2+} complex, provided 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 then freeze dried (Fig. 1A).

2.3. Monosaccharide composition

Each polysaccharide fraction (1 mg) was hydrolyzed with 2 M TFA at 100 °C for 8 h, followed by evaporation to dryness. The residues were successively reduced with $NaBH_4$ (1 mg) and acetylated with Ac_2O-pyridine (1:1, v/v; 200 μL) at 100 °C for 30 min following the method of Sassaki et al. (2008). The resulting alditol acetates were analyzed by gas chromatography–mass spectrometry (GC–MS), using a Varian model 3 300 gas chromatograph linked to a Finnigan Ion-Trap, Model 810-R12 mass spectrometer. Incorporated was a DB-225 capillary column (30 m × 0.25 mm i.d.) programmed from 50 to 220 °C at 40 °C min^{-1}, then hold, and the alditol acetates identified by their typical retention times and electron impact profiles.

2.4. Determination of homogeneity of β-D-glucans and their molecular weight (M_w)

The homogeneity and molar mass (M_w) of water-soluble purified glucan fraction FSHW 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^{-1} NaN_3. The solutions were filtered through a membrane, with pores of 0.22 μm diameter (Millipore). The specific refractive index increment (dn/dc) was determined, using a Waters 2 410 detector, the samples being dissolved in the eluent, five increasing concentrations, ranging from 0.2 to 1.0 mg mL^{-1} being used to determine the slope of the increment.

2.5. Methylation analysis of β-glucans

Per-*O*-methylation of each isolated polysaccharide (10 mg) was carried out using NaOH-Me_2SO-MeI as described by Ruthes, Komura, Carbonero, Sassaki, Gorin, and Iacomini (2010). The process, after isolation of the products by neutralization (HOAc), dialysis, and evaporation was repeated, and the methylation was found to be complete. The per-*O*-methylated derivatives were hydrolyzed with 45% aqueous formic acid (1 mL) for 15 h at 100 °C (Carbonero et al., 2012), followed by $NaBD_4$ (sodium borodeuteride) reduction and acetylation as above (item 2.3), to give a mixture of partially *O*-methylated alditol acetates, which was analyzed by GC–MS using a CP-Sil-43CB capillary column as described at item 2.3, and identified from m/z of their positive ions, by comparison with standards, the results being expressed as a relative percentage of each component (Sassaki, Gorin, Souza, Czelusniak, & Iacomini, 2005).

2.6. Controlled smith degradation of β-glucans

The purified glucans (IHW and FSHW; 100 mg each) were each submitted to oxidation with 0.05 M aqueous $NaIO_4$ (15 mL) at room temperature for 72 h in the dark. Ethylene glycol was added to stop the reaction, the solutions dialyzed, and the resulting polyaldehydes were reduced with $NaBH_4$ for 24 h, neutralized with HOAc, dialyzed, and concentrated (Goldstein, Hay, Lewis, & Smith, 2005). The residues were partially hydrolyzed with TFA pH 2.0 (30 min at 100 °C); followed by dialysis against tap water using membranes with a size exclusion of 2 kDa and the solutions containing retained material were freeze-dried. An aliquot (40 mg) of each degraded fraction, SM-IHW and SM-FSHW, was submitted to ^{13}C NMR spectroscopy and a sample (10 mg) was submitted to methylation analysis.

FSHW (500 mg) was further submitted to three cycles of controlled Smith degradation as described above to produce a linear polysaccharide (SM_3-FSHW) formed only by its main chain. This was compared for biological potential to those of isolated β-glucans, IHW and FSHW.

2.7. Nuclear magnetic resonance (NMR) spectroscopy

^{13}C NMR spectra were obtained using a 400 MHz Bruker model DRX Avance spectrometer incorporating Fourier transform. Analyses were performed at 70 °C on samples dissolved in D_2O (FSHW) or Me_2SO-d_6 (IHW and SM derived fractions). Chemical shifts of water-soluble samples are expressed in δ (ppm) relative to acetone at δ 30.20 and 2.22 for ^{13}C and ^{1}H signals, respectively, and at δ 39.70 (^{13}C) and 2.40 (^{1}H) for those dissolved in Me_2SO-d_6.

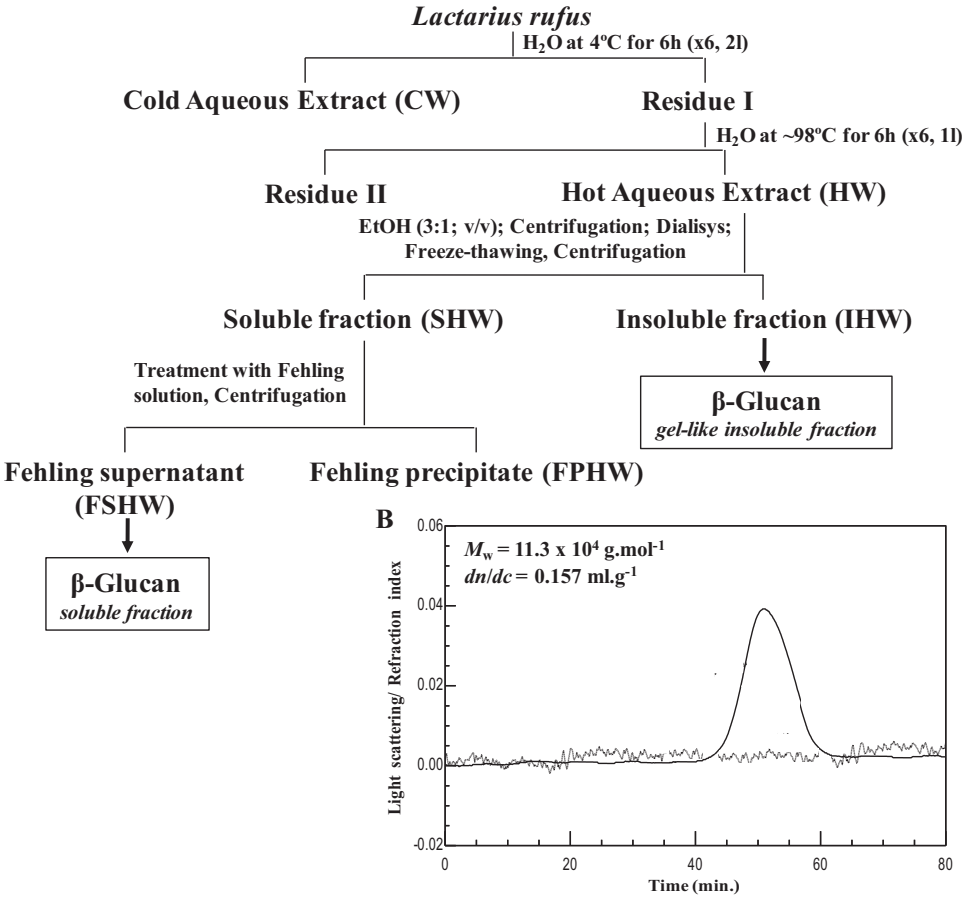

Fig. 1. (A) Scheme of extraction and purification of β-glucans from *L. rufus* (IHW and FSHW); (B) elution profile of fraction FSHW determined by HPSEC using light scattering (—) and refractive index detectors (—).

2.8. Experimental animals

Experiments were conducted using Swiss mice of both sexes (25–35 g) provided from Universidade Federal de Santa Catarina (UFSC) facilities, were kept in an automatically controlled temperature room (20 ± 2 °C) on a 12 h light–dark cycle (light on from 6:00 a.m.), with food and water being freely available. Animals (male and female mice were homogeneously distributed among groups) were acclimatized to the laboratory for at least 2 h before testing and were used only once for experiments. These were performed after approval of the protocol by the Institutional Ethics Committee of the Universidade Federal de Santa Catarina and in accordance with current guidelines for the care of laboratory animals and ethical guidelines for investigation of experimental pain in conscious animals (Zimmermann, 1983). The number of animals and intensities of noxious stimuli used were the minimum necessary to demonstrate consistent effects of the drug treatments.

2.9. Nociception induced by intraplantar injection of formalin

The procedure used was essentially the same as that described previously (Santos & Calixto, 1997). Animals received 20 μL of a 2.5% formalin solution (0.92% formaldehyde in saline), injected intraplantarly in the ventral surface of the right hind paw. Animals were observed from 0 to 5 min (early phase) and 15–30 min (late phase). Mice were treated with different doses of FSHW, IHW or SM_3-FSHW (1–30 mg kg^{-1}) by the intraperitoneal route 30 min beforehand. Control animals received a similar volume of the saline solution (10 mL kg^{-1}, i.p.), used to dilute the FSHW, SM_3-FSHW or vehicle (saline plus 5% Tween 80) for fraction IHW. After the intraplantar injection of formalin, animals were immediately placed in a glass cylinder 20 cm in diameter. The time they spent licking the injected paw was recorded with a chronometer and considered as indicative of nociception.

2.10. Statistical analysis

The results are presented as mean ± standard error of the mean (SEM), except for the ID_{50} values (*i.e.*, the dose of polysaccharide necessary to reduce the nociceptive response by 50% relative to the control value), which were reported as geometric means accompanied by their respective 95% confidence limits. The ID_{50} value was determined by nonlinear regression from individual experiments using linear regression GraphPad software (GraphPad software, San Diego, CA, USA). The statistical significance of differences between groups was detected by ANOVA followed by Newman–Keuls' test. *P*-values less than 0.05 ($p < 0.05$) were considered to be indicative of significance.

3. Results

3.1. Structural characterization of (1→3),(1→6)-β-D-glucans

In order to obtain pure polysaccharides from the fruiting bodies, *L. rufus* was submitted to successive cold and hot aqueous extraction at 4 °C (Ruthes, Rattmann, Carbonero, Gorin, & Iacomini, 2012) and ~98 °C, respectively (Fig. 1A). This report will present only results obtained from hot aqueous extraction, once polysaccharide fractions obtained from cold aqueous extraction are described in a previous publication (Ruthes et al., 2012). Polysaccharides were

Table 1
Partially *O*-methylated acetates formed on methylation analysis of the β-D-glucans obtained from *L. rufus* fruiting bodies: linkage types.

Partially *O*-methylated alditol acetates[a]	*Rt*[b]	% Area of fragments[c]				Linkage type
		FSHW	SM-FSHW	IHW	SM-IHW	
2,3,4,6-Me_4-Glc	22.66	32.0	6.8	20.5	Tr[d]	Glc*p*-(1→3→)-Glc*p*-(1→3,6→)-Glc*p*-(1→
2,4,6-Me_3-Glc	24.45	36.4	86.7	59.1	99.8	
2,4-Me_2-Glc	25.05	31.6	6.5	20.3	–	

[a] GC–MS analysis on a CP-Sil-43CB capillary column.
[b] Retention time (min).
[c] Based on derived *O*-methylalditol acetates.
[d] Trace.

recovered from hot aqueous extracts by ethanol precipitation, followed by centrifugation and dialysis of the precipitate against tap water. The solution was then freeze-dried to give rise to fraction HW (Fig. 1A).

Fractionation and purification of HW crude extract was carried out by a freeze–thawing procedure (Gorin & Iacomini, 1984), resulting in the respective cold water-soluble SHW (1.63 g, 3.9%) and a gel-like insoluble fraction, IHW (0.195 g, 0.5%).

Fraction SHW gave heterogeneous HPSEC elution profile; therefore it was purified by treatment with Fehling solution. The respective soluble fraction FSHW (0.560 g, 1.3%) showed to be homogeneous on HPSEC, with M_w 1.13×10^5 g mol^{-1} (dn/dc 0.157 mL g^{-1}) (Fig. 1B). While the water-insoluble IHW can be assumed as purified fraction based on its chemical composition, ^{13}C NMR and methylation data.

Each purified polysaccharide fraction contained only glucose as its monosaccharide component (GC–MS). Analysis by GC–MS of their partially *O*-methylated alditol acetates suggested the existence of two branched (1→3),(1→6)-linked β-D-glucans due to the presence of 2,3,4,6-Me_4-Glc, 2,4,6-Me_3-Glc, and 2,4-Me_2-Glc, determined in molar ratio of 1:1.2:1 (FSHW) and 1:3:1 (IHW), respectively (Table 1). These data reflect the difference observed on solubility among the fractions, showing that soluble β-D-glucan is more branched than the insoluble one.

Fractions FSHW and IHW were examined using NMR spectroscopy and signals were assigned using 1D (^{1}H, ^{13}C, and DEPT-135) and 2D NMR spectra (^{1}H (obs.) ^{13}C HMQC and COSY). All the signals were compared to literature values for similar polysaccharides (Carbonero et al., 2006, 2012; Smiderle et al., 2006; Smiderle, Olsen, Carbonero, Baggio, et al., 2008;

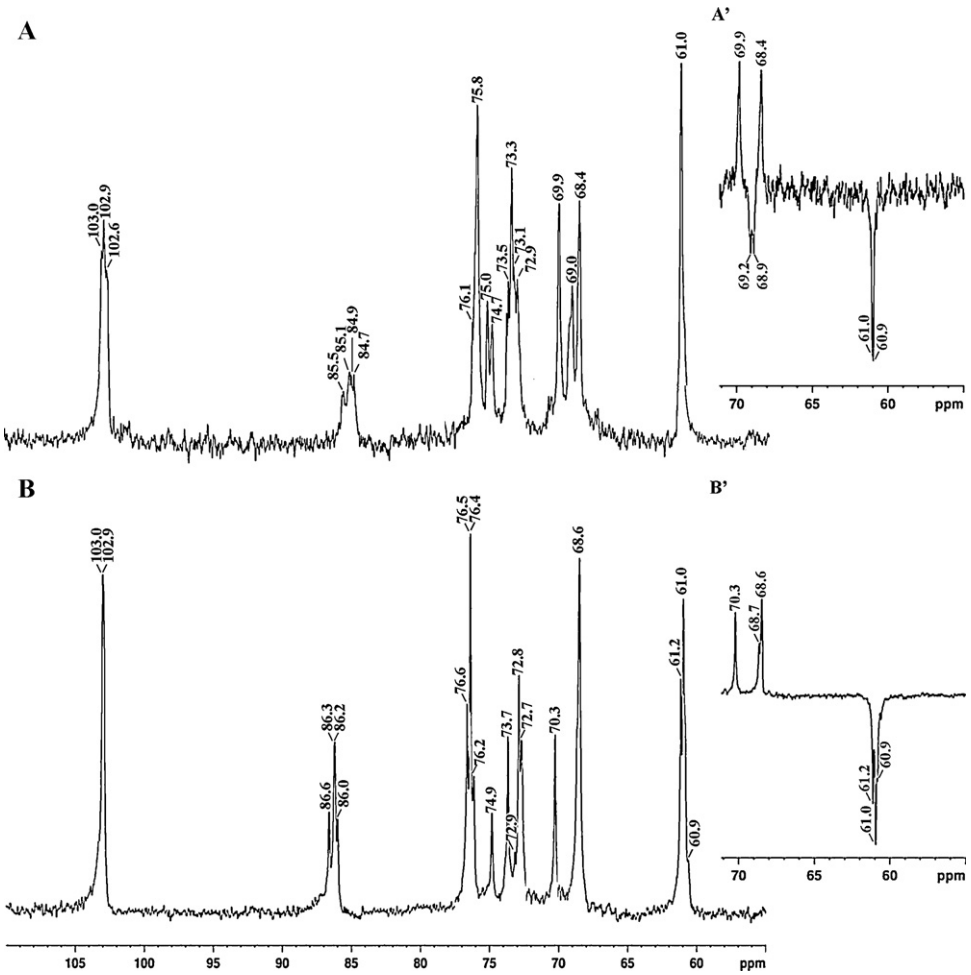

Fig. 2. ^{13}C NMR spectra of glucan fractions, FSHW (A) and IHW (B) with inserts of DEPT —$\underline{C}H_2$ inversion (A′ and B′, respectively); β-D-glucans, in D_2O (A) and Me_2SO-d_6 (B) at 70 °C (chemical shifts are expressed in δ ppm).

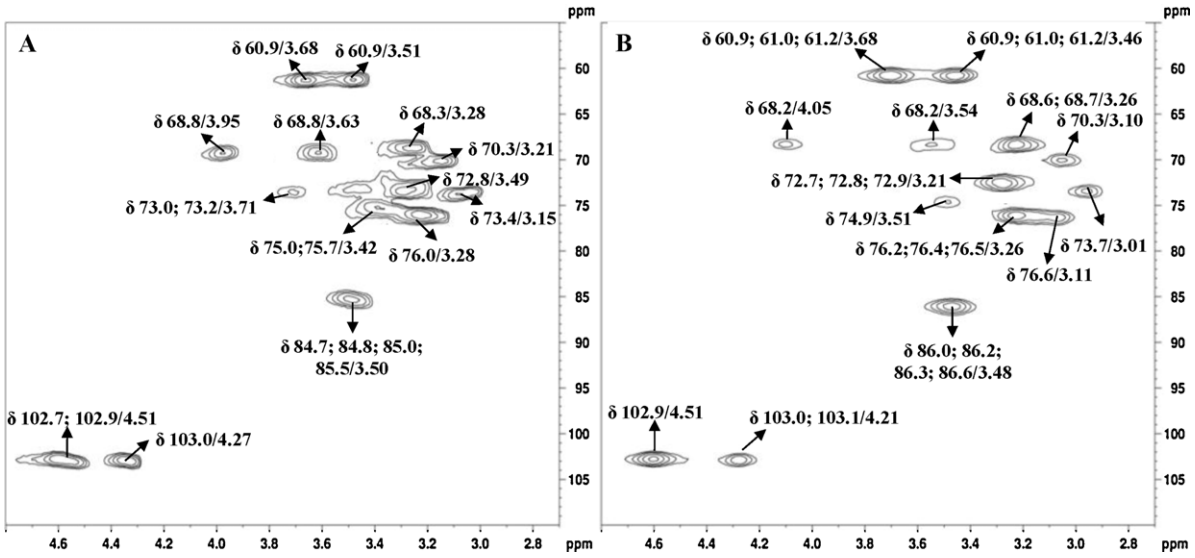

Fig. 3. ^{1}H (obs.)/^{13}C HMQC spectra of FSHW (A) and IHW (B) of β-D-glucan fractions in D_2O (A) and Me_2SO-d_6 (B) at 70 °C (chemical shifts are expressed in δ ppm).

Tabata, Ito, & Kojima, 1981; Yoshioka, Tabeta, Saito, Uehara, & Fukuoka, 1985).

^{13}C NMR (Fig. 2) and ^{1}H (obs.), ^{13}C HMQC spectra (Fig. 3) of β-D-glucan obtained from soluble fraction FSHW (Figs. 2A and 3) contained three distinct signals in the anomeric region at δ 103.0/4.30, 102.9/4.51 and 102.7/4.56, corresponding to non-reducing end, 3-*O*- and 3,6-di-*O*-substituted residues, respectively. While for the insoluble gel-like fraction (IHW) only two signals could be assigned at δ 103.1/4.21 corresponding to non-reducing end units, and at δ 102.9/4.51 from 3-*O*- and 3,6-di-*O*-substituted residues (Figs. 2B and 3). The β-configuration was confirmed by the 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) (Figs. 2 and 3) (Hall & Johnson, 1969).

The glycosidic linkages of these glucans were shown by the presence of 3-*O*-substituted signals at δ 85.4, 85.1 and 84.9 for FSHW (Fig. 2A) and at δ 86.6, 86.3, and 86.0 for IHW (Fig. 2B). The O-6 substitution was confirmed from the respective inverted peak in the DEPT-135 spectra (Fig. 2A′ and B′). *O*-Substituted C-6 signals downfield at δ 68.8 (FSHW) and at δ 68.2 (IHW-Lr) appeared as a doublet in HMQC at δ 68.8; 3.98/3.66 (Fig. 3A) and at δ 68.2; 4.05/3.54 (Fig. 3B), respectively.

The backbone structures of the two isolated glucans were characterized by controlled Smith degradation, and their residual polysaccharide products (SM-FSHW and SM-IHW, respectively) were analyzed by ^{13}C NMR (Fig. 4) spectroscopy and ^{1}H (obs.) ^{13}C HMQC (data not shown). It can be assumed that each fraction has a linear main-chain formed by (1→3)-linked β-D-glucan, due to the presence of typical C/H signals 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-FSHW), low intensity signals from O-6 substitution were still present (Fig. 4A), showing that this glucan is mostly substituted at O-6 by single-units of β-D-Glc*p* and a minor proportion by side chains of 3-*O*-substituted β-D-glucopyranosyl oligosaccharides.

The products of controlled Smith degradation were analyzed by methylation, and resulting alditol GC–MS analysis gave almost exclusively a 2,4,6-Me_3-glucitol derivative for IHW (99.8%; Table 1), indicating that the substitution occurs by single-units of glucose. These results further confirm that the non-reducing end units of β-D-Glc*p*, attached to the main-chain, were completely oxidized by periodate. On the other hand, and confirming the NMR data, the soluble fraction contained not only substitutions by nonreducing end-units, but it was also substituted by (1→3)-linked β-D-Glc*p* side chains (Table 1). To identify the extension of the few side chains, FSHW was submitted to a new round of controlled Smith degradation. Three cycles of oxidation were required for their complete removal to produce a linear polysaccharide formed only by its main chain (SM_3-FSHW), so probably, the side chains have a maximum of three (1→3)-linked β-D-Glc*p* units. This structure was further used to compare its biological potential to those of isolated β-glucans, IHW and FSHW, obtained from *L. rufus*.

The results therefore suggest that the insoluble glucan (IHW) is very similar to the purified glucans from *Boletus erythropus* (Chauveau, Talaga, Wieruszeski, Strecker, and Chavant, 1996), *Pleurotus florida* (Santos-Neves et al., 2008), *Pleurotus eryngii* and *Pleurotus ostreatoroseus* (Carbonero et al., 2006), *Pleurotus ostreatus* (Yoshioka et al., 1985), *Pleurotus pulmonarius* (Smiderle, Olsen, Carbonero, Baggio, et al., 2008) and also from *Flammulina velutipes* fruiting bodies (Smiderle et al., 2006). In contrast, the water-soluble glucan (FSHW) appears to be different from other mushroom (1→3),(1→6)-linked β-D-glucans.

In conclusion, the results of monosaccharide composition, methylation data, NMR spectroscopic analysis, and controlled Smith degradation of the studied fractions showed the presence of β-D-glucans with a (1→3)-linked main chain. These can be partially substituted at O-6 by non-reducing end units of β-D-Glc*p* as side-chains, on the average to every fourth residue of the backbone for the insoluble one (IHW) or partially substituted at O-6 mostly by single-units of β-D-Glc*p* and a minor proportion by 3-*O*-substituted β-D-glucopyranosyl oligosaccharides for soluble sample (FSHW).

3.2. *Effect of FSHW, IHW and SM_3-FSHW on nociception induced by intraplantar injection of formalin*

It has been demonstrated that different polysaccharides isolated from mushrooms display a variety of biological activities, including antinociceptive and anti-inflammatory effects. Among these are heterogalactans and glucans (Carbonero et al., 2008; Komura et al., 2010; Queiroz et al., 2010; Smiderle, Olsen, Carbonero, Baggio, et al., 2008; Ruthes et al., 2013).

The formalin test is a model of sustained peripheral damage frequently used to search for drugs with antinociceptive or anti-inflammatory activities (Tjølsen, Berge, Hunskaar, Rosland, & Hole, 1992). In addition, the injection of formalin gives rise to an early

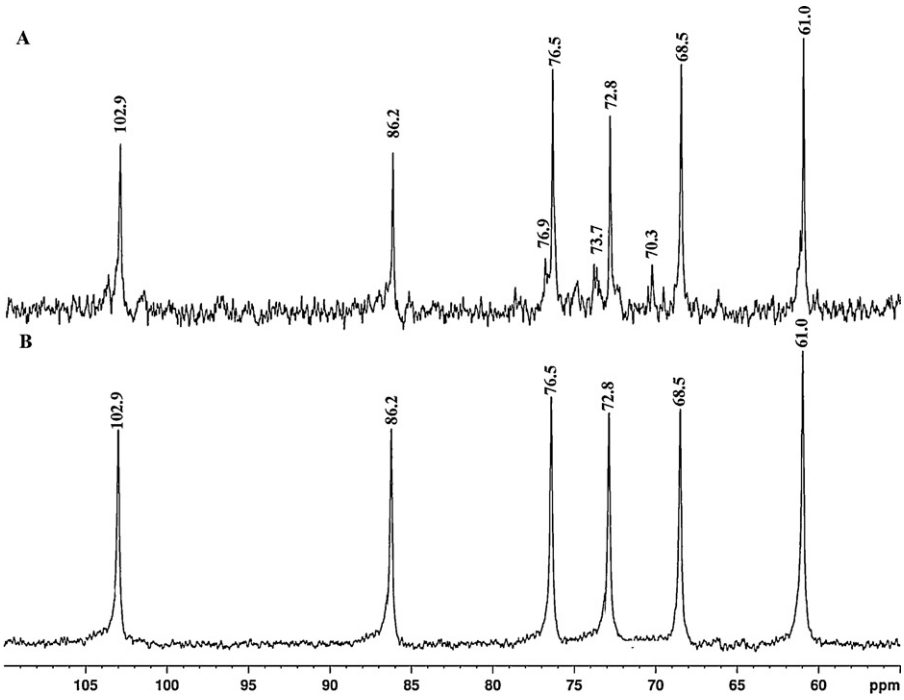

Fig. 4. ^{13}C NMR spectra of Smith-degraded glucans from *L. rufus* (SM-FSHW and SM-IHW; A and B, respectively), in Me_2SO-d_6 at 70 °C (chemical shifts are expressed in δ ppm).

phase (1–5 min after injection, approximately) and a late phase (15–60 min approximately) of pain sensation in humans and nociceptive behavior in animals (Porro & Cavazzuti, 1993; Tjølsen et al., 1992). Furthermore, spinal nociceptive neurons with receptive fields in the injected paw show corresponding early and late phases of firing (Dickenson & Sullivan, 1987). The early phase of the formalin response is due to the excitation of spinal cord neurons by a barrage of afferent impulses. The late phase is due to the effect of lingering afferent impulses upon spinal neurons that have now become hyper excitable due to the release of neuroactive mediators, and is thus regarded as a model of central sensitization (Hunskaar & Hole, 1987; Porro & Cavazzuti, 1993; Tjølsen et al., 1992). Thus, it has been shown that injection of formalin evokes a significant increase in the spinal cord concentration of neuropeptides, excitatory amino acids (glutamate and aspartate), nitric oxide, and pro-inflammatory mediators (Porro & Cavazzuti, 1993; Tjølsen et al., 1992).

We now, in order to evaluate the biological action of the β-glucans isolated from *L. rufus*, tested them on the nociception induced by formalin, an inflammatory pain model, which has a good correlation with the pain that occurs in humans. The results demonstrated that intraperitoneal administration of FSHW, IHW and SM_3-FSHW, at doses of 30 mg kg^{-1}, reduced the neurogenic pain (early phase) with inhibition of 36 ± 8%, 47 ± 13%, and 58 ± 4%, respectively (Fig. 5A, C and E). However, these polysaccharide samples were more effective against inflammatory pain (late phase) of formalin-induced nociception, with inhibition of 99 ± 1%, 96 ± 3%, and 80 ± 9%, respectively (Fig. 5B, D and F). Moreover, it is significant that the polysaccharide fraction, FSHW, which is soluble and more branched, was significantly more potent than IHW (insoluble glucan and less branched) and SM_3-FSHW (modified glucan, with linear structure) to inhibit the late phase of formalin-induced nociception (Fig. 5B, D and F), with ID_{50} values of 2.35 (1.48–3.75), 10.83 (9.27–12.63) and 10.56 (6.54–17.04) mg kg^{-1}, respectively. On the other hand, also at doses of 30 mg kg^{-1} exactly the contrary occurred in the early phase, relative to neurogenic phase (Fig. 5A, C and E), where linear β-glucan (SM_3-FSHW) had a major inhibitory effect, when compared to IHW and FSHW glucans, with intermediary and greater branching degree, respectively.

The neurogenic pain inhibition observed for IHW (47 ± 13%), showed to be very close to that observed for a structurally similar β-glucan isolated from *P. pulmonarius* (43 ± 5%) also at 30 mg kg^{-1} (Smiderle, Olsen, Carbonero, Baggio, et al., 2008). The same comparison could be done with the late phase results, where IHW inhibit 96 ± 3% formalin-induced nociception, as well as observed for *P. pulmonarius* β-glucan (96 ± 4%) (Smiderle, Olsen, Carbonero, Baggio, et al., 2008). Such a relationship cannot be undertaken for the soluble glucan (FSHW) or the linear product obtained after three cycles of controlled Smith degradation (SM_3-FSHW), since these structures are being first described. Thus, they were not previously assessed for their potential as possible biological response modifiers.

4. Discussion

β-Glucans have been extensively studied regarding their isolation and structural characterization. Such research showed remarkable biological properties, mostly antitumor, attributed to these polysaccharides. Hot water extracts from edible mushrooms have been used for a long time in traditional oriental medicine, as antitumor, anti-infective and anti-inflammatory drugs. Later research established that such biological activity is mainly due to the presence of β-glucans having common structure of (1→3)-linked backbone with single glucosyl units attached to the main-chain through (1→6)-glycosidic linkage (Kogan, 2000).

As biological response modifiers, β-glucans action is mediated through the organism's own defense tools. Such host-mediated defense is advantageous in comparison with the traditional approach of using synthetic or semi-synthetic therapeutics, once β-glucans are non-toxic to the host organism cells (Kogan, 2000).

Some studies have indicated that the distribution of the single glucosyl units along the main chain confers their immunomodulating activity, with their solubility in water also being important for biological applications (Bohn & BeMiller, 1995). Thus, the soluble

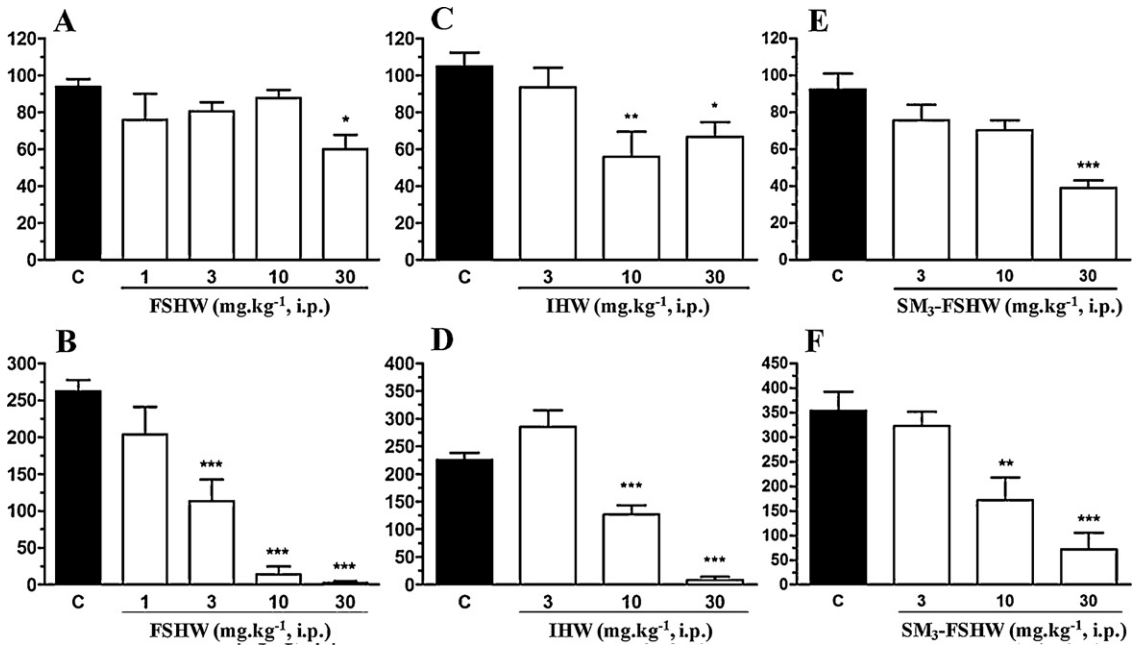

Fig. 5. Effects of intraperitoneally administered of FSHW, IHW or SM_3-FSHW on formalin-induced nociception (early phase, panels A, C and E; and late phase, panels B, D and F) in mice. Each column represents the mean 4–6 animals ± S.E.M. Control values (*C*) indicate the animals injected with saline 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).

β-D-glucan of *L. rufus* could be considered as a good candidate for evaluation of analgesic and anti-inflammatory potential.

Several research groups have tested mushroom extracts having anti-inflammatory and antinociceptive effects (Diyabalanage, Mulabagal, Mills, DeWitt, & Nair, 2008; Dore et al., 2007; Lin, Chen, Chiang, & Lin, 2006; Liu et al., 2007; Moro et al., 2012; Park et al., 2005; Queiroz et al., 2010; Wu, Duan, Liu, & Cen, 2010). However, the bioactive molecule(s) could not be identified. Studies evaluating such effects with pure mushroom polysaccharides are still few. Heterogalactans are the most studied polysaccharides regarding to antinociceptive and anti-inflammatory potential (Carbonero et al., 2008; Komura et al., 2010; Ruthes et al., 2013; Smiderle, Olsen, Carbonero, Marcon, et al., 2008).

P. pulmonarius (1→3),(1→6)-linked β-glucan, which showed anti-inflammatory and analgesic properties (Baggio et al., 2010, 2012; Smiderle, Olsen, Carbonero, Baggio, et al., 2008) contain the closest structure to which obtained results could be related, once its chemical structure is very similar to that of the β-glucan characterized from fraction IHW.

However, the soluble glucan is now shown to have a structure with a higher degree of branching, having not only being substituted by single Glc*p* units, but also by some (1→3)-linked Glc*p* side chains, making it different from all other glucans already having their biological potential evaluated.

The formalin test was chosen for being a widely used model of persistent pain and a mainstay for novel drug development for the treatment of postoperative pain (Shields et al., 2010). The test can be divided into two distinct phases. The first is characterized by neurogenic pain caused by direct chemical stimulation of nociceptors. The second is characterized by inflammatory pain triggered by a combination of stimuli, including peripheral tissue inflammation and mechanisms of central sensitization (Tjølsen et al., 1992). In the formalin test, FSHW, IHW and SM_3-FSHW showed a greater inhibition in the second phase than in the first, suggesting that its antinociceptive effect is related to inflammatory pain.

Therefore, our results demonstrate that the β-glucans (FSHW, IHW and SM_3-FSHW) tested have an activity similar to those of non-steroidal anti-inflammatory, once they did not produced profound effects on the first phase of intraplantar injection of formalin, but showed significant inhibition in the second phase response (Malmberg & Yaksh, 1992; Smiderle, Olsen, Carbonero, Baggio, et al., 2008).

Furthermore, it was clearly observed that *L. rufus* β-glucans branching degree and mainly the polysaccharides sample solubility seems to be important for their antinociceptive effect in response for both, neurogenic and inflammatory pain, but inversely. In the future, more experiments are necessary to identify the specific mechanism behind this inhibition of inflammation.

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