Carbohydrate Polymers 94 (2013) 91–99

Contents lists available at SciVerse ScienceDirect

Carbohydrate Polymers

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

Agaricus bisporus and *Agaricus brasiliensis* (1 → 6)-β-D-glucans show immunostimulatory activity on human THP-1 derived macrophages

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ARTICLE INFO

Article history:
Received 20 September 2012
Received in revised form
23 November 2012
Accepted 31 December 2012
Available online 16 January 2013

Keywords:
Agaricus bisporus
Agaricus brasiliensis
(1 → 6)-β-D-Glucan
Immunostimulatory activity

ABSTRACT

The (1 → 6)-β-D-glucans from *Agaricus bisporus* and *Agaricus brasiliensis* were purified to evaluate their effects on the innate immune system. THP-1 macrophages were used to investigate the induction of the expression of TNF-α, IL1β, and COX-2 by RT-PCR. The purification of the polysaccharides gave rise to fractions containing 96–98% of glucose. The samples were analyzed by GC–MS, HPSEC and ^{13}C NMR, which confirmed the presence of homogeneous (1 → 6)-β-D-glucans. The β-glucans were incubated with THP-1 derived macrophages, for 3 h and 6 h to evaluate their effects on the expression of pro-inflammatory genes. Both β-glucans stimulated the expression of such genes as much as the pro-inflammatory control (LPS). When the cells were incubated with LPS + β-glucan, a significant inhibition of the expression of IL-1β and COX-2 was observed for both treatments after 3 h of incubation. By the results, we conclude that the (1 → 6)-β-D-glucans present an immunostimulatory activity when administered to THP-1 derived macrophages.

1. Background

Mushrooms or mushroom polysaccharide preparations have been extensively studied as immune modulators and adjuvant agents in cancer treatment. These fungi have traditionally been used for the prevention and treatment of a multitude of disorders, and they have been increasingly consumed by cancer patients, during their treatments, as dietary supplements (Hardy, 2008; Moradali, Mostafavi, Ghods, & Hedjaroude, 2007). Among the mushroom extracts, the most used are derived from *Trametes versicolor*, *Grifola umbellatae*, *Ganoderma lucidum*, *Agaricus brasiliensis*, and *Lentinus edodes*. All of these are rich in β-glucans (Hardy, 2008; Wasser, 2002). These polymers are the basis of fungal cell wall structure. *In vitro* experiments showed that β-glucans can directly activate leukocytes and other immune system cells, stimulating their phagocytic, cytotoxic, and antimicrobial activity (Brown & Gordon, 2003). In addition, they can stimulate the production of pro-inflammatory mediators, such as cytokines and chemokines (Chanput, Mes, Vreeburg, Savelkoul, & Wichers, 2010), and act as antitumor (Ren, Perera, & Hemar, 2012), anti-oxidative (Toklu et al., 2006), anti-inflammatory (Dore et al., 2007), and immunomodulatory (Lull, Wichers, & Savelkoul, 2005) compounds.

Bioactive polysaccharides, including β-glucans, are recognized by membrane receptors of leukocytes and macrophages, as CR3, dectin-1 and TLRs, leading to proliferation and differentiation of immune cells (Li & Xu, 2011; Lull et al., 2005; Moradali et al., 2007). These activities are responsible for enhancing the innate and cell-mediated immune responses, with the expression of pro-inflammatory genes and consequently, for the induction of antitumoral and bactericidal effects (Ramberg, Nelson, & Sinnott, 2010; Schepetkin & Quinn, 2006).

Differences have been reported for immunostimulating and antitumoral activities of β-(1 → 3)-glucans when compared to β-(1 → 4)- and β-(1 → 6)-glucans. This may be explained by physical-chemical differences, although the relationship between structure and medicinal property requires more investigation (Lehtovaara & Gu, 2011; Ren et al., 2012; Thompson, Oyston, & Williamson, 2010). Their primary structure, molecular weight, branching frequency, and quaternary structure are important for the bioactivity of these compounds. For instance, lentinan, the (1 → 3)–(1 → 6)-β-D-glucan isolated from Shiitake (*Lentinus edodes*), in its triple-helical conformation was found to inhibit the growth of solid tumors (sarcoma-180). This inhibitory effect was not observed when the tumors were treated by the single-helical polysaccharide (Zhang, Li, Xu, & Zeng, 2005). Studies have shown

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http://dx.doi.org/10.1016/j.carbpol.2012.12.073

that soluble and insoluble β-glucans are able to induce the production of cytokines by specific immune cells. Nevertheless it still remains unclear how the molecular weight of β-glucans relates to their overall biological activity (Ren et al., 2012). Molecular weight does affect binding efficiency to dectin, one of the β-glucan receptors (Palma et al., 2006), with a size of minimally 10- or 11-glucose residues being required for binding. Since other receptors (e.g. TLR-2, TLR-4, CR3, lactosylceramide, scavenger receptors) can also recognize β-glucans (Netea, Brown, Kullberg, & Gow, 2008), and simultaneous binding to multiple receptors may alter the cytokine response (Meyer-Wentrup, Cambi, Adema, & Figdor, 2005), optimal binding to a single receptor is not necessarily equivalent to optimal physiological effect.

Macrophages and dendritic cells (DC's) play important roles in many host reactions. They absorb and process antigen material and present it on their surface to other cells of the immune system, i.e. to the T-helper cells. Macrophages might behave both as pro-inflammatory cells to prevent e.g. infectious disease, as well as anti-inflammatory cells with a reparatory effect, as in wound healing (Mosser & Edwards, 2008). By binding to their receptors, the bioactive polysaccharides activate various immune pathways like phagocytosis, complement activity, and respiratory burst and also the production of cytokines such as tumor necrosis factor-α (TNF-α), different kinds of interleukins (IL's) and enzymes as cyclooxygenase-2 (COX-2) (Huang et al., 2012; Lull et al., 2005; Thompson et al., 2010). All these effects collaborate to modulate cell differentiation and proliferation, enabling the host to defend itself against pathogens and induction of tumors.

The monocytic THP-1 human myeloid leukemia cell line expresses the Fc receptor and can be induced by phorbol 12-myristate 13-acetate (PMA) to differentiate into macrophage-like morphology (Kohro et al., 2004; Tsuchiya et al., 1980). Upon PMA treatment the suspension culture changes and cells become adherent to glass and plastics and show an increase in mitochondrial and lysosomal numbers and in cytoplasmic to nuclear ratio, as well as an altered differentiation dependent on cell surface markers in a pattern similar to monocyte-derived macrophages (Daigneault, Preston, Marriott, Whyte, & Dockrell, 2010). In addition they have a high absorption capacity for latex micro beads and express a cytokine profile that resembles macrophages.

In our previous study we determined the carbohydrate composition of semi-purified extracts from 2 closely related and well known basidiomycete species, i.e. *Agaricus bisporus* and *Agaricus brasiliensis*. Both extracts presented mixtures of three main polysaccharides: (1 → 4)-(1 → 6) α-D-glucan, (1 → 6)-β-D-glucans, and mannogalactan. *A. brasiliensis* showed higher contents of (1 → 6)-β-D-glucans (49.1%) while *A. bisporus* presented mannogalactan (55.8%) as the main polysaccharide. However the extracts showed similar effects when tested on THP-1 (Smiderle et al., 2011). We now decided to continue the study by the purification of the (1 → 6)-β-D-glucan from both mushrooms to compare their effects on the innate immune system, using the same model (THP-1 macrophages) to investigate the induction of the expression of pro-inflammatory genes.

2. Methods

2.1. Fungal material

Fresh fruiting bodies of cultivated *A. bisporus* (strain 7215 – Mycelia, Belgium) and *A. brasiliensis* (strain M7700 – Mycelia, Belgium) were produced by Makoto Yamashita firm, São José dos Pinhais, PR, Brazil.

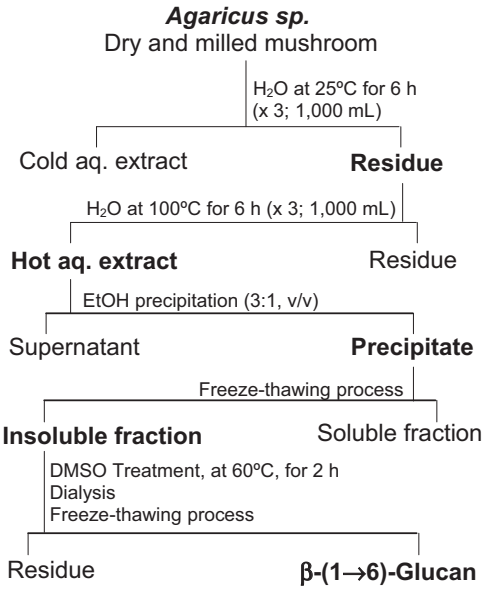

Fig. 1. Extraction and purification of the β-D-glucans.

2.2. Extraction and purification of the β-D-glucans

The dried mushrooms (*A. bisporus*, 100 g; *A. brasiliensis*, 50 g) were processed, separately, using the same extraction procedures as shown in Fig. 1. Both were lyophilized, milled and submitted to successive cold and hot aqueous extraction, successively, for 6 h (×3; 1,000 mL each). The cold extraction was performed aiming to separate other compounds, as phenols, heteropolysaccharides, and glycogen, which were not the focus of this study.

The hot aqueous extracts from each mushroom were evaporated to a small volume and the polysaccharides were precipitated by addition to excess ethanol (3:1; v/v) and centrifuged at 10,000 rpm, at 10 °C, for 20 min. The sediment was dialyzed against tap water for 24 h (12–14 kDa cut-off), concentrated under reduced pressure and freeze-dried. *A. bisporus* and *A. brasiliensis* extracts were named HW-Ab and HW-Abz, respectively. The purification was performed by freeze-thawing process (Gorin & Iacomini, 1984): fractions HW-Ab and HW-Abz were dissolved in water and the solutions were submitted to freeze and thaw slowly until complete separation of soluble and insoluble polysaccharides. The precipitates (P-HW-Ab; P-HW-Abz), obtained after centrifugation (10,000 rpm, at 4 °C, for 20 min), were treated with dimethylsulfoxide (50 mL), for 2 h, at 60 °C, dialyzed against tap water for 24 h and then resubmitted to the freeze-thawing process, giving rise to soluble fractions of β-D-glucans (Glc-Ab and Glc-Abz). The purified polysaccharides were tested on THP-1 cells.

2.3. Analysis of monosaccharide composition by GC–MS

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 maintained at 100 °C for 10 min, in order to reduce aldoses to alditols (Sassaki, Gorin, Souza, Czelusniak, & Iacomini, 2005). The product was dried and excess $NaBH_4$ was neutralized by the addition of acetic acid or 1 M TFA (100 μL), which was removed following the addition of methanol (2×) under a N_2 stream in a fume hood. Acetylation of the Me-alditols was performed in pyridine–Ac_2O (200 μL; 1:1, v/v), heated for 30 min at

100 °C. The resulting alditol acetates were analyzed by GC–MS, and identified by their typical retention times and electron impact profiles. Gas liquid chromatography–mass spectrometry (GC–MS) was 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 et al., 2005).

2.4. Detection of LPS contamination of the polysaccharide samples by GC–MS

The LPS standard from *Escherichia coli* serotype O111:B4 was obtained from Sigma (St. Louis, MO, USA) and diluted in deionized water and the solution was then sonicated (two cycles of 15 min). An aliquot of LPS (0.3 mg) was collected and placed in reinforced Pyrex tubes with 4 mL Teflon lined screw-cap vessels (Supelco, Bellefonte, PA, USA). The solution was dried under a N_2 stream, and the residue was dissolved in 400 μL MeOH (Merck, Darmstadt, Germany) and subjected to methanolysis by the addition of 100 μL of 3 M MeOH–HCl (Supelco, Bellefonte, PA, USA). The vial was vortexed (1 min) and kept at 80 °C for 20 h. The solution was then partitioned between hexane (1 mL) and deionized water (0.5 mL). The hexane phase was evaporated under a gentle N_2 stream, and acetylated with a mixture of pyridine (100 μL) and acetic anhydride (100 μL) at 100 °C for 1 h. Serial dilutions of the acetylated product were then prepared, using acetone as solvent, corresponding to concentrations of 75, 50, 37.5, 25, and 10 ng of LPS, which were analyzed by GC–MS. Each sample (Glc-Ab/Glc-Abz, 1 mg/mL) was diluted in deionized water and vigorously shaken for 12 h. An aliquot containing 0.3 mg of each sample was then collected, and processed as described above up to the acetylation step. Samples were gently evaporated under a N_2 stream, and the residues dissolved in acetone (5 μL), concentrated down to 1 μL, and analyzed by GC–MS as described in details by Santana-Filho et al. (2012).

2.5. Methylation analysis

Per-*O*-methylation of each isolated polysaccharide (10 mg) was carried out using NaOH-Me_2SO-MeI (Ciucanu & Kerek, 1984). After isolation of the products by neutralization (HOAc), dialysis, and evaporation, the methylation process was repeated. The per-*O*-methylated derivatives were hydrolyzed with 45% aqueous formic acid (1 mL) for 15 h at 100 °C, followed by NaB^2H_4 reduction and acetylation as above, 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 et al., 2005).

2.6. Nuclear magnetic resonance spectroscopy

NMR spectra were obtained using a 400 MHz Bruker Avance III spectrometer with a 5 mm inverse probe. ^{13}C NMR (100.6 MHz) analyses were performed at 70 °C in D_2O or DMSO-d_6, chemical shifts being expressed in δ ppm relative to external standard of acetone (δ 30.2 for ^{13}C signal) or DMSO-d_6 (δ 39.7 for ^{13}C signal), respectively.

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

The homogeneity and molar mass (M_w) of the purified glucan fractions Glc-Ab and Glc-Abz were 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 (M_w) of each polymer was estimated using Astra software 4.70.

2.8. Cell culture

The human monocytic cell line THP-1 (Rio de Janeiro Cell Bank, Rio de Janeiro, Brazil) was grown in RPMI 1640 culture medium (Sigma–Aldrich, cat. R8758) supplemented with 10% heat-treated newborn calf serum Sterile A (Gibco, cat. 161010-159) and 100 U/mL resp. 100 μg/mL penicillin/streptomycin (P/S) (Sigma–Aldrich), at 37 °C in 5% CO_2 in a humidified incubator. The medium was renewed twice a week.

2.9. Macrophage differentiation and stimulation

The mature macrophage-like state was induced by treating THP-1 monocytes (500,000 cells/mL) for 48 h with 30 ng/mL phorbol 12-myristate 13-acetate (PMA; Sigma–Aldrich) in 24-wells polystyrene tissue culture plates (Costar) with 1 mL cell suspension in each well. The medium was then removed and replaced by fresh medium containing the isolated β-glucans (Glc-Ab and Glc-Abz) at 100, 50, and 25 μg/mL; or phosphate buffered saline (PBS; 50 μL), or lipopolysaccharide (LPS; 1 μg/mL) as negative and pro-inflammatory controls, respectively. Cells were harvested at the time points 0 h, 3 h, and 6 h and kept in lysis buffer at −20 °C for the next step. Time point 0 h was used to normalize the calculations. All experiments were performed with the same amount of cells (0.5×10^6 per mL). The total RNA was isolated from the cells as follows.

2.10. Gene expression kinetics by real-time PCR

Total RNA was isolated by using RNeasy mini kit (Qiagen, USA) with a RNase-free DNase (Qiagen) treatment for 15 min according to the manufacturer's instructions. Complementary DNA (cDNA) was synthesized from isolated RNA (1 μg) with High Capacity RNA-to-cDNA kit (Applied Biosystems, USA). Expression levels of each gene were measured in triplicate reactions, performed with the same cDNA pool (1:5 diluted), in the presence of the fluorescent dye (iQ SYBR Green Supermix) using an StepOne Plus™ instrument (Applied Biosystems, USA). The experiments were performed in a 20 μL reaction volume with specific primer pairs (Chanput et al., 2010), and the conditions of real-time quantitative PCR were as follows: denaturation at 95 °C for 10 min and amplification by cycling 40 times at 95 °C for 15 s and 60 °C for 60 s. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), β-actin (ACTB), and β-2-microglobulin were used as endogenous control, and GAPDH was chosen for normalization. The PCR of all products were subjected to a melting curve analysis to verify the single amplification product. The relative messenger RNA (mRNA) expression were presented as described in Chanput et al. (2010): the values were expressed as fold change relative to the value at time point zero, calculated as $\Delta\Delta Ct\,[\Delta\Delta Ct = 2\hat{(}Ct_{GAPDH} - Ct_{Sample})]$ (Livak & Schmittgen, 2001). The q-PCR analyses were performed twice on each sample (in triplicate), to evaluate the mRNA expression level of pro-inflammatory cytokine genes IL-1β and TNF-α and also the inflammation-related enzyme COX-2.

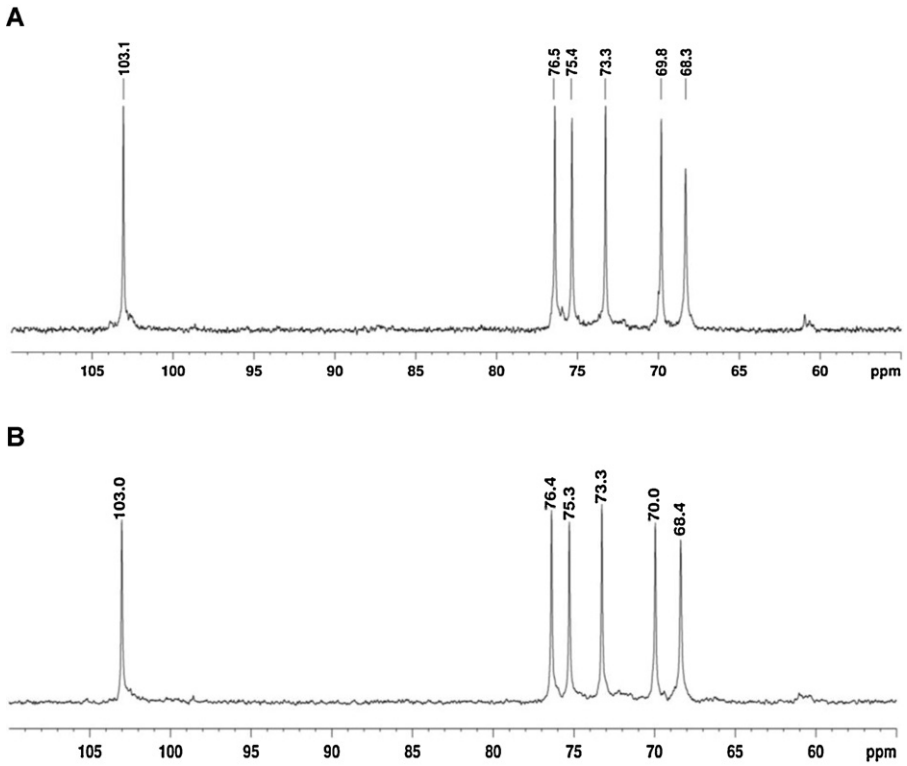

Fig. 2. ^{13}C NMR spectra of *A. bisporus* (1 → 6)-β-D-glucan (A), and *A. brasiliensis* (1 → 6)-β-D-glucan (B). ^{13}C NMR experiments were performed in D_2O at 50 °C (chemical shifts are expressed in δ ppm).

3. Statistical analysis

The results are expressed as mean ± standard deviation of duplicate cultures of two representative experiments. Statistical significance was determined using one-way analysis of variance (ANOVA) followed by Bonferroni's test, selected pairs. $p \leq 0.05$ was considered statistically significant. The graphs were drawn and the statistical analyses were performed using GraphPad Prism version 5.01 for Windows (GraphPad Software, San Diego, CA, USA).

4. Results

Crude extracts from *A. bisporus* and *A. brasiliensis* were studied previously (Smiderle et al., 2011) and both showed immunomodulatory effects on THP-1 cells. Both extracts contained β-D-glucans, as described before (Ohno et al., 2001; Smiderle et al., 2011), which were purified now to verify the immunomodulatory properties of the isolated (1 → 6)-β-D-glucans.

4.1. Chemical structure of the purified β-D-glucans

The β-D-glucans were isolated from the fruiting bodies of each mushroom, using hot water extractions, as described previously. After freeze-thawing process, partial precipitation took place and soluble polysaccharides (S-HW-Ab, 2,0 g; S-HW-Abz, 0,3 g) were separated from the insoluble ones (P-HW-Ab, 2,3 g; P-HW-Abz, 0,6 g) by centrifugation (Fig. 1). The monosaccharide composition showed that the soluble fractions consisted of mannose, galactose, and glucose, while the insoluble fractions showed mainly glucose, suggesting the presence of β-glucans. These fractions were submitted to a purification procedure, by treatment with DMSO (50 mL), at 60 °C, for 2 h. The DMSO-soluble materials were recovered after centrifugation, being dialyzed and lyophilized. The obtained purified samples were analyzed by GC–MS, showing 98% (Glc-Ab) and 96% (Glc-Abz) of glucose for *A. bisporus* and *A. brasiliensis*, respectively.

The NMR spectroscopy of samples Glc-Ab and Glc-Abz confirmed the presence of a linear β-glucan (1→6)-linked in each sample, by the presence of only six signals as shown in the ^{13}C NMR spectra (Fig. 2). All carbon frequencies were assigned according to the literature (Barreto-bergter and Gorin, 1983), and the β-configuration was confirmed by the high frequency of carbon-1 (δ 103.1 and 103.0 ppm, respectively) (Hall & Johnson, 1969). The other carbon frequencies were assigned as C-2 (δ 73.3/73.3 ppm), C-3 (δ 76.5/76.4 ppm), C-4 (δ 69.8/70.0 ppm), C-5 (δ 75.4/75.3 ppm), and C-6 (δ 68.3/68.4 ppm), as shown in Fig. 2. The *O*-substituted $-CH_2$ (C-6) signals were confirmed by their inversion in DEPT-^{13}C NMR spectra (data not shown). Both (1 → 6)-β-D-glucans showed homogeneous elution profiles when analyzed by HPSEC (Fig. 3A). Their molecular weights were estimated at 2.9×10^4 g/mol (Glc-Ab) and 4.5×10^4 g/mol (Glc-Abz). A scheme of the isolated (1 → 6)-β-D-glucans is presented in Fig. 3B.

Both glucans were checked whether there was LPS contamination by GC–MS (as described in material and methods) showing less than 5 ng of LPS/100 μg of Glc-Ab, and no LPS was detected on Glc-Abz.

In our previous study (Smiderle et al., 2011), we observed that semi-purified polysaccharide extracts from *A. bisporus* and *A. brasiliensis* stimulated the expression of pro-inflammatory genes (IL-1β, TNF-α and COX-2) by THP-1 cells. Both extracts contained three main polysaccharides: α-glucan, β-glucan, and galactomannan in different proportions. The immunomodulatory effects observed in THP-1 cells were quite similar for both extracts. In this study, we isolated the (1 → 6)-β-D-glucans of each species to evaluate the immunomodulatory properties of this particular molecule.

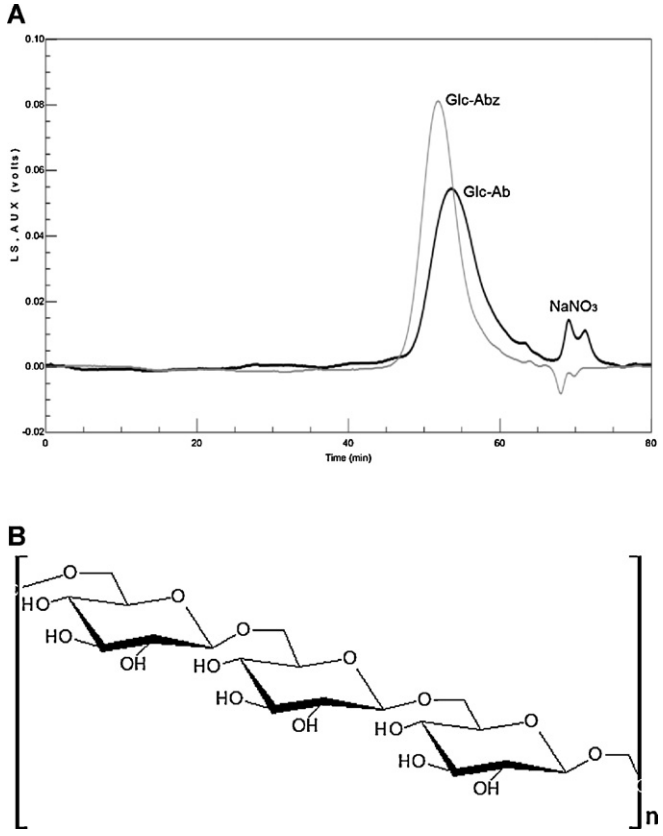

Fig. 3. Elution profiles of fractions Glc-Ab and Glc-Abz determined by HPSEC using refractive index detectors, and 0.1 M $NaNO_3$ as eluent (A). Scheme of the structure of the (1 → 6)-β-D-glucan isolated from both *Agaricus spp.* (B).

4.2. Immunomodulatory activity

The results observed for the β-glucan isolated from *A. bisporus* (Glc-Ab) showed a significant expression ($p \leq 0.05$) of the pro-inflammatory genes after 3 h of treatment (Fig. 4). Although the lower concentration (25 μg/mL) showed the lower effect, the expression of IL-1β and TNF-α, observed for the higher concentrations, were comparable or even higher than the pro-inflammatory control (LPS) stimulation. IL-1β was the only gene which expression was stimulated even after 6 h of treatment.

The β-glucan isolated from *A. brasiliensis* (Glc-Abz) was also tested in the same concentrations and it showed significant stimulation of the expression of IL-1β and COX-2 after 3 h and 6 h of treatment (Fig. 5). TNF-α was poorly expressed, its highest value being observed at 100 μg/mL of β-glucan, after 3 h.

The crude polysaccharide extracts from these mushrooms were able to reduce the pro-inflammatory effects caused by the bacterial toxin LPS (i.e. increase in the expression of IL-1β and TNF-α), when THP-1 macrophages were stimulated with this endotoxin (Smiderle et al., 2011). The β-glucan from *A. bisporus* (Glc-Ab) was able to reduce or inhibit the expression of IL-1β to 40%, at 50 μg/mL, after 3 h of treatment, compared to the pro-inflammatory control (LPS) (Fig. 6). Expression of COX-2 was reduced 50% or more, after 3 h of treatment for all doses. However this effect was not observed for TNF-α, which was highly expressed after 3 h of treatment in any condition.

The β-glucan from *A. brasiliensis* (Glc-Abz) also significantly reduced or inhibited the expression of IL-1β and COX-2 (Fig. 7), when the cells were stimulated by LPS concurrently. The highest dose of β-glucan showed a reduction of 64% for IL-1β, and 36% for COX-2. The concentration of 50 μg/mL reduced the expression

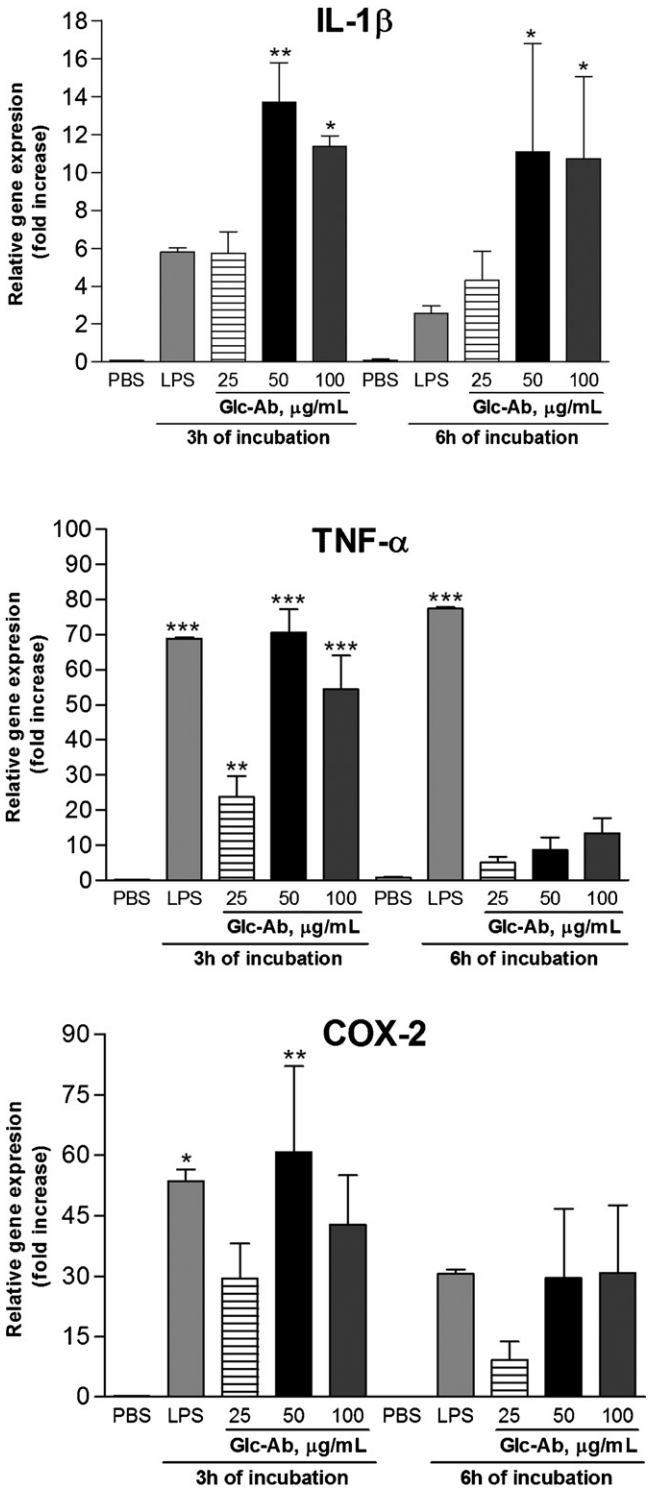

Fig. 4. mRNA expression level of genes for IL-1β, TNF-α, and COX-2 after treatment with *A. bisporus* β-D-glucan for 3 h and 6 h.
Legend: Negative control (PBS), pro-inflammatory control (LPS; 1 μg/mL), Glc-Ab100 (100 μg/mL), Glc-Ab50 (50 μg/mL), and Glc-Ab25 (25 μg/mL). Statistical analyses were performed by means of one-way analysis of variance (ANOVA) followed by Bonferronis' test, selected pairs. The results represent the mean ± SD of duplicate cultures of two representative experiments. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$ versus negative control.

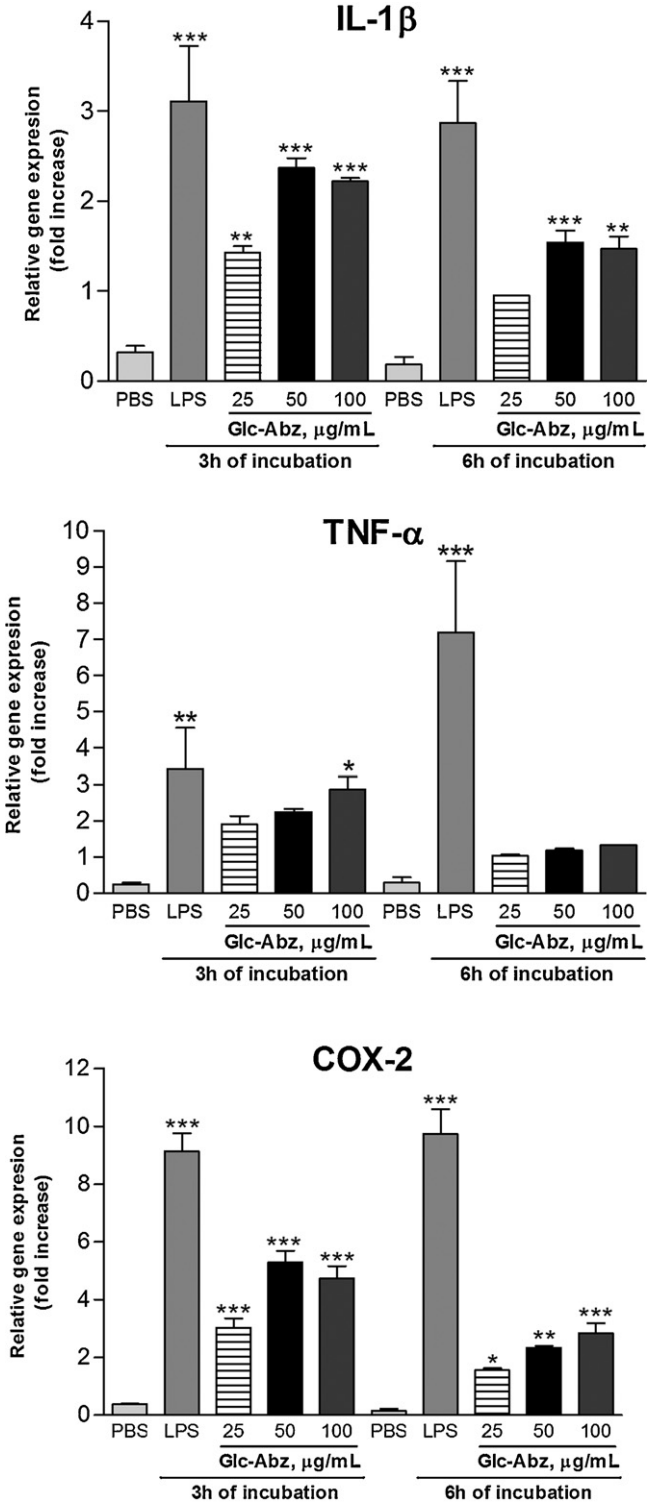

Fig. 5. mRNA expression level of genes for IL-1β, TNF-α, and COX-2 after treatment with *A. brasiliensis* β-D-glucan for 3 h and 6 h.
Legend: Negative control (PBS), pro-inflammatory control (LPS; 1 μg/mL), Glc-Abz100 (100 μg/mL), Glc-Abz50 (50 μg/mL), and Glc-Abz25 (25 μg/mL). Statistical analyses were performed by means of one-way analysis of variance (ANOVA) followed by Bonferronis' test, selected pairs. The results represent the mean ± SD of duplicate cultures of two representative experiments. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$ versus negative control.

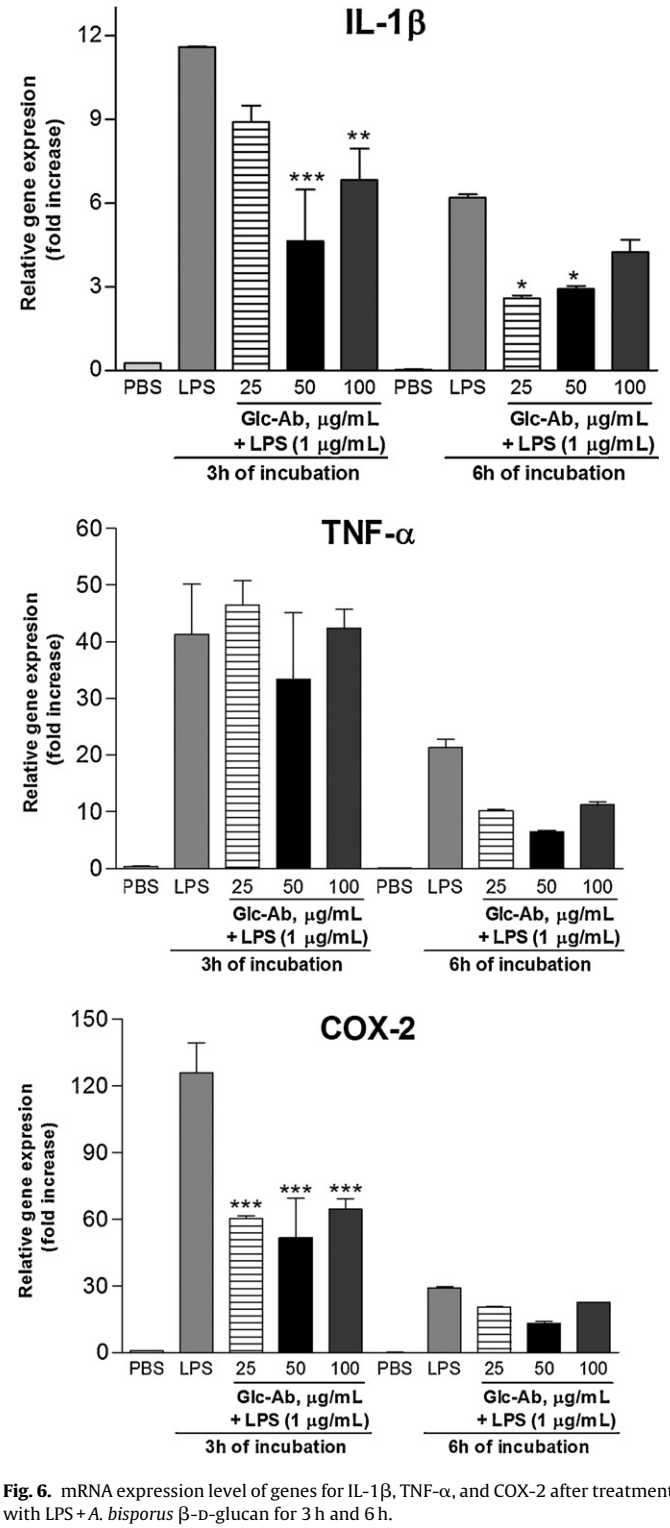

Fig. 6. mRNA expression level of genes for IL-1β, TNF-α, and COX-2 after treatment with LPS + *A. bisporus* β-D-glucan for 3 h and 6 h.
Legend: Negative control (PBS), pro-inflammatory control (LPS; 1 μg/mL), Glc-Ab100 + LPS (100 μg/mL + 1 μg/mL), Glc-Ab50 + LPS (50 μg/mL + 1 μg/mL), and Glc-Ab25 + LPS (25 μg/mL + 1 μg/mL). Statistical analyses were performed by means of one-way analysis of variance (ANOVA) followed by Bonferronis' test, selected pairs. The results represent the mean ± SD of duplicate cultures of two representative experiments. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$ versus pro-inflammatory control.

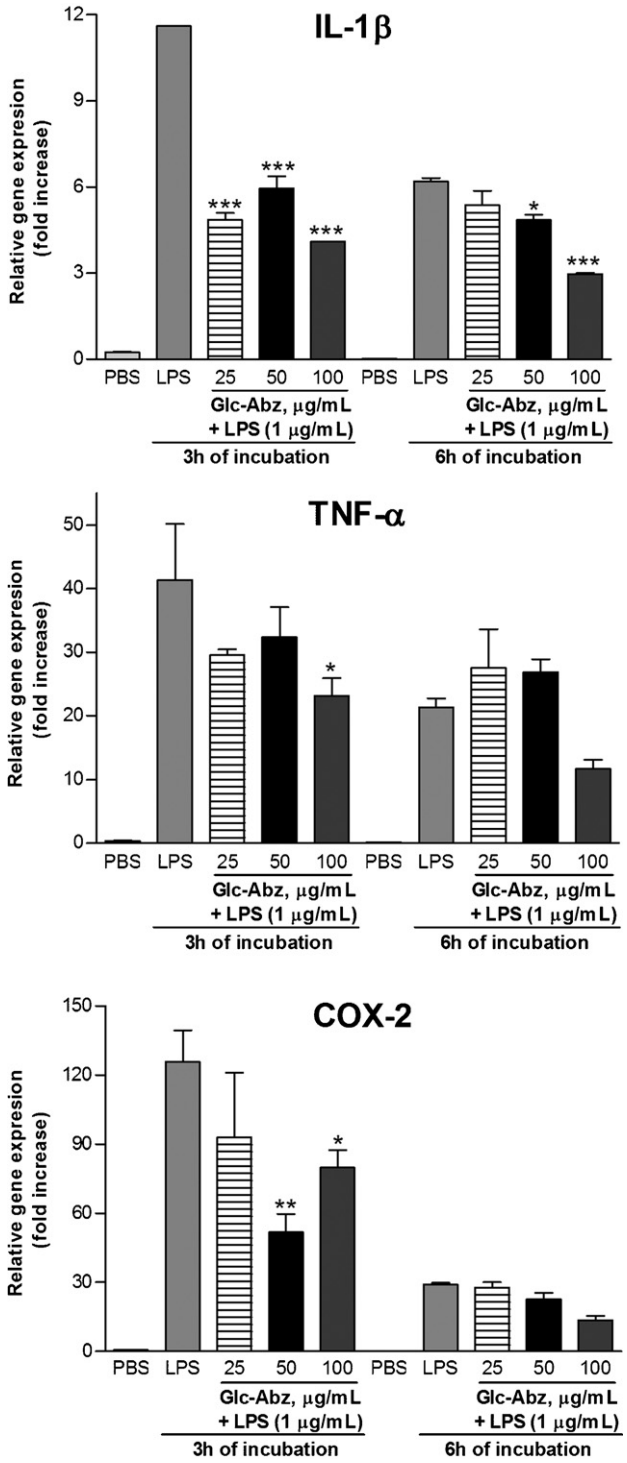

Fig. 7. mRNA expression level of genes for IL-1β, TNF-α, and COX-2 after treatment with LPS + *A. brasiliensis* β-D-glucan for 3 h and 6 h.
Legend: Negative control (PBS), pro-inflammatory control (LPS; 1 μg/mL), Glc-Abz100 + LPS (100 μg/mL + 1 μg/mL), Glc-Abz50 + LPS (50 μg/mL + 1 μg/mL), and Glc-Abz25 + LPS (25 μg/mL + 1 μg/mL). Statistical analyses were performed by means of one-way analysis of variance (ANOVA) followed by Bonferronis' test, selected pairs. The results represent the mean ± SD of duplicate cultures of two representative experiments. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$ versus pro-inflammatory control.

of COX-2 by 59% compared to the pro-inflammatory control (LPS). TNF-α expression was diminished only at 100 μg/mL, after 3 h, while no effect was observed for the lower doses.

5. Discussion

Basidiomycetes are known to present medicinal properties, which are being attributed to their glucans and other polysaccharides. The most common glucans extracted from these organisms are (1→3), (1→6)-β-D-glucans (Ren et al., 2012; Schepetkin & Quinn, 2006), although linear β-(1→3)- or β-(1→6)-glucans are also encountered, as well as α-glucans (Lindequist, Niedermeyer, & Jülich, 2005; Smiderle et al., 2010). In the previous study (Smiderle et al., 2011), we compared the polysaccharide extracts from *A. bisporus* and *A. brasiliensis*, which showed comparable NMR profiles. Both extracts contained mixtures of three main polysaccharides and their compositions were similar to what had been observed for other basidiomycete mushrooms, i.e. containing glucose, galactose, mannose and fucose (Ohno et al., 2001). *A. brasiliensis* showed higher contents of (1→6)-β-D-glucan (49.1%) while *A. bisporus* contained mannogalactan (55.8%) as the main polysaccharide. The proportion of each polysaccharide encountered for both species varied significantly, and this may be an explanation for the differences observed in the biological effects described by other authors. *A. brasiliensis* is known to be a medicinal mushroom and it has been widely used in Japan for many years for the treatment of cancer and other diseases (Camelini et al., 2005). *A. bisporus*, on the other hand, is highly consumed as food; however there is quite some evidence concerning its possible therapeutic and preventive properties in reduction of blood glucose (Yamac et al., 2010) cholesterol levels (Jeong et al., 2010), as well as anticancer activity (Chen et al., 2006).

In the present study, we decided to isolate the (1→6)-β-D-glucan from both *Agaricus* species and test their immunomodulatory properties, with the aim to continue the investigation on both mushrooms and compare the effects of each β-D-glucan on macrophages. For this purpose, THP-1 monocytes were differentiated into macrophages, which were incubated with the isolated (1→6)-β-D-glucans. The expression of pro-inflammatory genes IL-1β, TNF-α, and COX-2 was measured, showing an increase comparable to the stimulation observed for the pro-inflammatory control (LPS). The effects observed for both β-D-glucans were not identical, although both were able to stimulate the expression of these genes, in some cases as high as the LPS stimulation. While Glc-Ab stimulated the expression of TNF-α at 25, 50, and 100 μg/mL, the Glc-Abz stimulated the expression of IL-1β and COX-2 at these concentrations, even after 6 h. TNF-α expression was not significant for both treatments, after 6 h of incubation, which can be explained by its short half-life, especially because TNF-α is one of the first cytokines to be produced during an inflammatory process (Sang, Wallis, Stewart, & Kotake, 1999; Sato, Keelan, & Mitchell, 2003).

The over-expression of pro-inflammatory genes, as TNF-α, can cause damage to the organism, considering that it is related to immunopathologies as arthritis and septic shock. The normal situation for an organism is to keep a balance between anti- and pro-inflammatory cytokines. When the cells, stimulated by LPS, were incubated with the β-D-glucans, a decrease of the expression of pro-inflammatory genes IL-1β and COX-2 was observed for both β-D-glucans. This reduction suggests that the (1→6)-β-D-glucan present an inhibitory effect on the LPS stimulus. The stimulation of pro-inflammatory cytokines by the (1→6)-β-D-glucan can be related to their binding to C-lectin type receptors, as dectin-1; and also to toll-like receptors (TLR-2, TLR-4, TLR-6). Polysaccharides from *Polyporus umbellatus* and *Cordyceps militaris* were shown to immunostimulate macrophages and dendritic cells, respectively, via TLR-4 signaling pathways (Kim et al., 2010; Li &

Xu, 2011). Therefore, the inhibition of IL-1β and COX-2 observed in Figs. 6 and 7 could be explained by a competition between LPS and the β-D-glucans, for binding to TLR-4 (Fujihara et al., 2003; Netea et al., 2008). Upon binding to LPS, TLR-4 activates both MyD88-dependent and TRIF-dependent signaling pahtways (Kim et al., 2010). Although the detailed events downstream of MyD88 and TRIF differ, MAPKs and NF-κB signaling are commonly activated, with subsequent expression of TNF-α, IL-1β, and COX-2 expression, among other genes (Moradali et al., 2007; Netea et al., 2008; Petrova et al., 2008). It has been proposed that the production of TLR-4-mediated inflammatory cytokines requires the cooperation of both signaling pathways (Fujihara et al., 2003). Besides, it has been shown that the downstream events of the MyD88-dependent pathway are mediated by three components: adaptor protein myeloid differentiation factor 88 (MyD88), a family of IL-1 receptor-associated kinases (IRAK), and another adaptor protein, TNF receptor-activated factor 6 (TRAF6). The inhibition of any of these molecules/pathways leads to a negative effect of the LPS-stimulation, with no expression of NF-κB and, consequently, of other pro-inflammatory genes (Burns et al., 2003; Fujihara et al., 2003; Janssens, Burns, Tschopp, & Beyaert, 2002). Calmodulin, a Ca^{2+}-dependent regulatory protein, could also have inhibited LPS-stimulated IL-1β expression (Ohmori & Hamilton, 1992). Consequently, the low levels of IL-1β would down regulate COX-2 expression (Clària, 2003), showing another possible interpretation of the results observed.

Another explanation could be the stimulation of anti-inflammatory genes by the β-D-glucans, as IL-10 for example, which exhibit inhibitory effects on the expression of TNF-α, leading to the reduction of other pro-inflammatory genes (Sato et al., 2003). As observed previously, simultaneous binding to multiple receptors may alter the cytokine response (Meyer-Wentrup et al., 2005). Considering that polysaccharides can bind to more than one membrane receptor, more insight of receptor-binding is needed. That will be the subject of our next study.

Prolonged incubation (more than 16 h) of cells (monocytes/macrophages) with sublethal doses of LPS induces a phenomenon named as endotoxin tolerance or LPS desensitization, which consists of a reduction of the LPS-inflammatory effects on the cells (Fujihara et al., 2003). This phenomenon was discarded as an explanation for the reduction of IL-1β and COX-2 expressions, considering that the THP-1 macrophages were incubated with LPS only for few hours (3 h–6 h), that was not enough to cause the desensitization (Janssens et al., 2002).

In our previous study the semi-purified polysaccharide of *A. brasiliensis* was able to reduce the expression of TNF-α induced by LPS. This was not observed when the cells were treated by the isolated (1 → 6)-β-D-glucan which leads to the conclusion that another component of the extract could possibly exhibit anti-inflammatory properties (Smiderle et al., 2011). This shows the importance of the purification and characterization of each compound present in the extracts to study their effect separately on the cells.

6. Conclusions

Both *A. bisporus* and *A. brasiliensis* contain a linear (1 → 6)-β-D-glucan, that can be isolated by DMSO extraction. The β-glucans of both species were found to stimulate the expression of pro-inflammatory genes when administered to THP-1 macrophages. Moreover, the β-glucans reduced the inflammatory effects of LPS when co-administered with this endotoxin, as shown by the inhibition of IL-1β and COX-2, the reduction of TNF-α was not significant. The mechanism by which the β-glucans act on the cells is still under debate.

Acknowledgments

The authors would like to thank to Emanuel M. de Souza and Valter A. Baura for the technical assistence with the RT-PCR analysis.

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