Antiviral properties of polysaccharides from Agaricus brasiliensis in the replication of bovine herpesvirus 1

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Summary. — Natural products are an inexhaustible source of compounds with promising pharmacological activities, including antiviral action. In the present study, the antiviral potential of polysaccharide-peptide (PLS) and an extracted β-glucan from Agaricus brasiliensis were investigated in the replication of bovine herpesvirus 1 (BoHV-1) in HEp-2 cell cultures. The cytotoxicity (CC₅₀) was assayed by the MTT method and the antiviral activity (IC₅₀) was estimated by the plaque reduction assay. To study the possible mode of action of PLS and β-glucan, the following protocols were performed: the virucidal assay, adsorption assay and the time-of-addition assay. The PLS presented a selectivity index (SI) higher than 12.50 and β-glucan 9.19. The antiviral inhibition (67.9%) in cells treated with PLS during virus infection was higher than that in cells treated prior to or post infection. The β-glucan presented high inhibition of virus replication by plaque assay (83.2%) and by immunofluorescence assay (63.8%). Although the mechanism has yet to be defined, we suggest that PLS and β-glucan inhibited BoHV-1 replication by interfering with the early events of viral penetration. Additional studies are required for a better understanding of the mechanism of action of PLS and β-glucan.

Keywords: bovine herpesvirus 1; Agaricus brasiliensis; antiviral activity

Introduction

The lack of effective therapies and/or vaccines for several viral infections and the emergence of drug-resistant strains have stimulated the research of new antiviral drugs. Natural products have proven to be an important source of both new pharmaceuticals and compounds that can be modified for improved efficacy (Ghosh et al., 2009). Among the fungi, molds have been a significant source of antimicrobial and immunomodulatory compounds. Higher fungi have been tested for antibacterial activity, but only recently has it been demonstrated that some mushrooms also possess antiviral activity. For example, a proteoglycan from the basidiomycete Ganoderma lucidum has antiviral activity against herpes simplex viruses (Li et al., 2005). An extract of the edible Japanese mushroom Lentinus edodes has been found to inhibit the replication of herpes simplex virus (HSV), western equine encephalitis virus, poliovirus, measles virus, mumps virus, vesicular stomatitis virus and human immunodeficiency virus (Tochikura et al., 1988; Sorimachi et al., 1990; Suzuki et al., 1993; Sasaki et al., 2001). Thus, the screening of other mushroom species may lead to the identification of additional antiviral drugs.

Agaricus brasiliensis (formerly Agaricus blazei), a native species from Brazil, popularly known as Sun Mushroom and Himematsutake in Japan, is thought to possess medicinal values in folk medicine. Infusion of the dried fruiting bodies of this mushroom has been popularly consumed both as a stimulant and as an auxiliary treatment for various diseases, including cancer (Pinto et al., 2009). Studies
showed that ethanol fractions obtained from an aqueous extract of *A. brasiliensis* mycelium completely inhibited the cytopathic effect of the western equine encephalomyelitis virus (Sorimachi *et al.*, 2001).

The extracts of *A. brasiliensis* contain a high amount of β-glucans, the class of polysaccharides that consist of a central chain of (β 1→6)-D-glucose with branches made of (β 1→3)-D-glucose. It has been shown that these compounds have antitumor and immunomodulatory properties (Kawagishi *et al.*, 1989; Dong *et al.*, 2002).

BoHV-1 belongs to the subfamily Alphaherpesvirinae and shares a number of biological properties with HSV-1 and HSV-2. The virus causes conjunctivitis, upper respiratory infection, pneumonia, genital disorders and abortions (Tikoo *et al.*, 1995). This disease represents annually a significant cost to the cattle industry. Although vaccines are available, they can cause abortions and disease in young calves (Jones, 2003).

As few reports exist investigating the antiviral activity of *A. brasiliensis*, the aim of the present study is to evaluate the antiviral potential of PLS and an extracted β-glucan from *A. brasiliensis* against BoHV-1.

**Materials and Methods**

*Polysaccharides.* *A. brasiliensis*-derived PLS obtained at LP/DQOI/UFC (Gonzaga *et al.*, 2005) was dissolved in Dulbecco’s Modified Eagle Medium (DMEM), filter-sterilized and kept at 4°C for a maximum of 72 hrs. The β-glucan was isolated from the polysaccharide-peptide assemblage extracted from *A. brasiliensis* (Angeli *et al.*, 2006) and supplied by Laboratório de Polímeros, Departamento de Química Orgânica e Inorgânica, UFC, Brazil. The compounds were dissolved in DMEM, submitted to sterilization by ultra-filtration in 0.2 μm pore size membrane, and stored at -20°C.

*Cells and virus.* HEp-2 cells (human larynx carcinoma, ATCC, CCL- 23) were grown at 37°C in DMEM supplemented with 10% fetal bovine serum (Gibco BRL, USA), 2 mmol/l glutamine*, 100 IU/ml penicillin*, 100 μg/ml streptomycin* (*Sigma, Chem. Co., USA) and 2.5 μg/ml amphotericin B (Bristol Myers-Squibb, Brazil). BoHV-1 was supplied by DMVP-UEL, Brazil. The virus was propagated in HEp-2 cells and stored at -80°C, and titers were determined by a plaque assay.

*Cytotoxicity assay.* For the cytotoxicity assay, HEp-2 cells were seeded in 96-well plates at a density of 10⁵ cells/ml. After 24 hrs, cultures were incubated with various concentrations (15–2,000 μg/ml of PLS and β-glucan for 48 hrs. Cell viability was determined by MTT kit (methyl-thiazolyl-diphenyl-tetrazolium bromide) (Sigma Chem. Co., USA) according to manufacturer’s instructions. The 50% cytotoxic concentration (CC₅₀) corresponding to the concentration of substances that reduces cell viability by 50% was calculated by linear regression analysis.

**Antiviral assay.** The inhibitory effect of PLS and β-glucan on BoHV multiplication was investigated by plaque reduction assay and immunofluorescence assay (IFA). HEp-2 cells seeded onto 24-well culture plates or glass coverslips were infected and treated with β-glucan (100 to 250 μg/ml) or PLS (50 to 400 μg/ml) and submitted to plaque reduction assay and Immunofluorescence assay as described below. The 50% inhibitory concentration (IC₅₀), defined as the concentration of substances required to reduce virus titer by 50%, was calculated by linear regression analysis. The SI was calculated by the ratio CC₅₀/IC₅₀. Interferon (human alpha-2 B, Meizler Com. Intern. SA, Brazil) at a concentration of 1000 U/ml was used as a positive control for inhibition of BoHV-1 replication.

**Plaque reduction assay.** Semi-confluent HEp-2 cell monolayers grown in 24-well plates were treated with PLS or β-glucan and after infection overlaid with nutrient agarose. Cultures were incubated for 48 hrs, fixed with 10% formalin and stained with 0.5% crystal violet. The antiviral activity was calculated as the percentage of plaque reduction as follows: % Plaque reduction = [1 – (Number of plaques in test/Number of plaques in control)] x 100.

*IFA to detect BoHV proteins.* was performed at test time of 0 hr after infection. HEp-2 cells grown on glass coverslips were infected with BoHV-1 at a MOI of 1. PLS (50 to 400 μg/ml) or β-glucan (100 to 250 μg/ml) were added at 0 hr, and the cells were incubated for 24 hrs. The cells were fixed with cold acetone ( -20°C) for 20 mins followed by immunofluorescence staining using polyclonal bovine anti-BoHV-1 antibodies (DMVP/UEL) and rabbit anti-bovine IgG FITC conjugate (Sigma Chem. Co., USA). The cells were examined under UV light (Leica DM 4500 B, Germany) and 100 cells/cover slips were scored.

**Time-of-addition assay of PLS.** The time-of-addition of the PLS in viral replication was done as previously described (Yang *et al.*, 2005). Semi-confluent HEp-2 cell cultures were incubated with PLS at concentrations of 50, 100, 200 or 400 μg/ml before (-1 hr and -2 hrs), during (0 hr) and after (1 hr and 2 hrs) infection, at 37°C under 5% CO₂. Cultures were infected with 50–100 PFU/ml of BoHV-1. The viral inhibition was monitored by plaque reduction assay 48 hrs after the infection.

**Virucidal assay.** BoHV-1 at 10⁶ PFU/ml was diluted with equal volumes of DMEM containing respectively 50, 100, 200, and 400 μg/ml of PLS, or 100, 200, and 250 μg/ml of β-glucan, followed by incubation for 1 hr at 37°C. Monolayers were incubated for 1 hr with virus plus substances, followed by washing and incubation, and plaque counting was performed after 48 hrs.

**Virus adsorption assay.** The virus adsorption assay was performed according to Zhu *et al.* (2004), with modifications. Cell monolayers were infected with BoHV-1 in the presence or absence of PLS or β-glucan at the concentrations previously mentioned. After a viral adsorption period of 1 hr at 4°C, the cells were washed with PBS to remove non-adsorbed virus, and after 48 hrs of incubation, plaques were counted.

**Statistics.** The statistical significance of the difference between mean values was determined by Student’s t-test at a significance level of P ≤0.05. The experiments were carried out in triplicate.
Results

Cytotoxicity of polysaccharides

The viability of HEp-2 cells was not affected by PLS even at the highest concentration used, i.e., 2,000 μg/ml. However, CC50 of the β-glucan in HEp-2 cells reached the value of 1,250 μg/ml.

Influence of time of addition of PLS on BoHV replication

HEp-2 cell monolayers were treated with PLS before, during or after virus infection, and the results demonstrated a concentration-dependent virus inhibition with the highest inhibitory effect in cell cultures treated at time 0 hr. Fig. 1 shows that PLS was more effective when present during the infection. When PLS was added at the concentration of 400 μg/ml, two hours or one hour prior to infection (-2 hrs and -1 hr), the highest inhibition of viral replication was 9.4% and 3.4%, respectively. At the same time, PLS added at time zero (0 hr) caused 67.9% inhibition. However, when PLS was added at 1 hr or 2 hrs post infection, inhibition was 42.9% and 28.6%, respectively. With PLS added at time zero at concentrations of 200 μg/ml, 100 μg/ml, and 50 μg/ml, inhibition were 58.5, 36.5, and 30.1%, respectively. When PLS was added before or after infection, the antiviral effect was lower (Fig. 1).

Effect of PLS and β-glucan on BoHV replication

The effect of PLS and β-glucan on BoHV-1 protein synthesis assayed by IFA is shown in Table 1. PLS concentrations of 400 μg/ml, 200 μg/ml, 100 μg/ml, and 50 μg/ml reduced the number of fluorescent cells (FC) by 61, 54, 38.8, and 29%, respectively. The IFA results demonstrated a concentration-dependent antiviral effect similar to that found for the plaque assay.

The antiviral activity of β-glucan on BoHV-1 replication monitored by plaque assay, at concentration of 250 μg/ml, 200 μg/ml, and 100 μg/ml, resulted in inhibition of 83.2, 72.4, and 37.8% (Fig. 2). When monitored at the same concentrations by IFA, the inhibition was 63.8, 56.5, and 39.1% (Table 1). The SI, determined as CC50/IC50, at time 0 hr and at the highest concentrations of PLS (400 μg/ml) and β-glucan (250 μg/ml) are shown in Table 2. The SI of PLS was higher than that of β-glucan, but both exhibited a good antiviral activity, with SI values higher than 12.50 and 9.19, respectively. The IC50 was calculated by regression analysis of the dose-response curve.

PLS and beta-glucan neither acted directly on virus particles (virucide protocol) nor affected virus attachment step. In both protocols there was no significant reduction in virus yield. Interferon used as control inhibited BoHV-1 replication by 100%.

Discussion

Current chemotherapeutic antiviral drugs have been characterized as having in many cases limited clinical efficacy, resistance and toxic side effects (Strasfeld and Chou, 2010). Therefore, it is necessary to identify and develop
fluorescent cells. FC = number of fluorescent cells; % FCI = percentage of inhibition of viral internalization, but preceding the onset of late viral protein synthesis (Gonzalez et al., 1994; Zhu et al., 1997). Aqueous and ethanol extracts and an isolated polysaccharide from the fruiting body of A. brasiliensis, also demonstrated higher antiviral activity against poliovirus type 1 in HEP-2 cells, when added just after virus inoculation, at time 0 hr (Faccin et al., 2007).

β-glucan demonstrated concentration-dependent antiviral against BoHV-1 at the time 0 hr of infection. This substance was isolated from a polysaccharide fraction derived from the fruiting body of A. brasiliensis, whose antiviral activity against poliovirus -1 was previously described by Faccin et al. (2007). The authors demonstrated that the polysaccharide was more effective when added at time 0 hr of infection in agreement, therefore with the results obtained in our study. Zhang et al. (2004) evaluated the β-glucans extracted from the fungus Pleurotus tuber-regium and found antiviral activity against HSV-1 and -2, where the inhibition was observed at time 0 hr of infection, similarly.

In conclusion, we demonstrated that PLS and the extracted β-glucan from A. brasiliensis possess antiviral activity against BoHV-1. The effective antiviral concentrations of PLS are far from the cytotoxicity threshold, and, consequently, this natural product shows a considerable selectivity index. Therefore, PLS as well as β-glucan can be used for the development of antiviral agents. The antiviral activity of PLS may be due to the effect at the stage of BoHV-1 penetration into host cell, as described for other polysaccharides, but the exact steps affected remain to be elucidated.

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References


A number of polysaccharides with antiviral activity have been described. Polysaccharides isolated from Antrodia camphorata have anti-hepatitis B virus activity (Lee et al., 1999). A protein-bound polysaccharide isolated from the mushroom Ganoderma lucidum was found to decrease significantly the viral titer of both HSV-1 and HSV-2 and the authors suggest that this polysaccharide impedes the complex interactions of viruses with cell, thus inhibiting the early stages of viral infection (Eo et al., 2000).

Antiviral activity of polysaccharides is linked to the anionic features of the molecules, resulting in inhibition of the early stages of viral infection such as attachment and penetration (Marchetti et al., 1995). Several sulfated polysaccharides, such as heparin, dextran sulfate, pentosan polysulfate, mannan sulfate, sulfated cyclodextrins, carrageenans, xylofuranan sulfate, and ribofuranan sulfate have been shown to inhibit the replication of various enveloped viruses, including HSV, human cytomegalovirus and human immunodeficiency virus (Damonte et al., 1994; Witvrouw et al., 1994; Zhu et al., 2006).

The results reported here are in agreement with the previous studies on the antiviral activity of polysaccharides, such as carrageenan. This polysaccharide inhibited a step in the HSV cycle following viral internalization, but preceding the onset of late viral protein synthesis (Gonzalez et al., 1987). Some anionic sulfate polysaccharides isolated from algae, such as rhamnose sulfate from Monostroma latissimum and fucose sulfate from Sargassum horneri, were found to inhibit the HSV-1 replication even when added at 2 hrs post infection, after some virus-specific proteins had already been synthesized (Hoshino et al., 1998; Lee et al., 1999).

<table>
<thead>
<tr>
<th>Concentrations (μg/ml)</th>
<th>PLS</th>
<th>β-glucan</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>59 ± 2</td>
<td>69 ± 3</td>
</tr>
<tr>
<td>50</td>
<td>42 ± 4</td>
<td>39.1 ± 3.1</td>
</tr>
<tr>
<td>100</td>
<td>36 ± 3</td>
<td>42 ± 2</td>
</tr>
<tr>
<td>200</td>
<td>27 ± 3</td>
<td>30 ± 3</td>
</tr>
<tr>
<td>250</td>
<td>----</td>
<td>25 ± 2</td>
</tr>
<tr>
<td>400</td>
<td>23 ± 1</td>
<td>63.8 ± 2.8</td>
</tr>
<tr>
<td></td>
<td>61.0 ± 2.5</td>
<td></td>
</tr>
</tbody>
</table>

The values represent the number of fluorescent cells with the respective standard deviation and corresponding percentage of inhibition. FC = number of fluorescent cells; % FCI = percentage of inhibition of fluorescent cells.

Table 2. The antiviral activity, expressed as SI, of PLS and β-glucan isolated from Agaricus brasiliensis on BoHV-1 in plaque reduction assay

<table>
<thead>
<tr>
<th>Substance</th>
<th>CC50 (μg/ml)</th>
<th>IC50 (μg/ml)</th>
<th>SF (CC50/IC50)</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-glucan</td>
<td>1250</td>
<td>140</td>
<td>9.19</td>
</tr>
<tr>
<td>PLS</td>
<td>&gt;2000</td>
<td>160</td>
<td>&gt;12.50</td>
</tr>
</tbody>
</table>

Fifty percent cytotoxic concentration. aFifty percent inhibitory concentration. Selectivity index.

SHORT COMMUNICATIONS


