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Protective effects of β-glucan extracted from *Agaricus brasiliensis* against chemically induced DNA damage in human lymphocytes

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Abstract

β-Glucans (BGs) are polysaccharides that are found in the cell walls of organisms such as bacteria, fungi, and some cereals. The objective of the present study was to investigate the genotoxic and antigenotoxic effects of BG extracted from the mushroom *Agaricus brasiliensis* (=*Agaricus blazei* Murrill ss. Heinemann). The mutagenic activity of BG was tested in single-cell gel electrophoresis assays with human peripheral lymphocytes. In addition, the protective effects against the cooked food mutagen 3-amino-1-methyl-5*H*-pyrido[4,3-*b*]indole (Trp-P-2) and (+/−)-anti-B[*a*]P-7,8-dihydrodiol-9,10-epoxide (BPDE), which is the main metabolite of B[*a*]P, and against ROS (H_2O_2)-induced DNA damage, were studied. The results showed that the compound itself was devoid of mutagenic activity, and that a significant dose-dependent protective effect against damage induced by hydrogen peroxide and Trp-P-2 occurred in the dose range 20–80 μg/ml. To investigate the prevention of Trp-P-2-induced DNA damage, a binding assay was carried out to determine whether BG inactivates the amine via direct binding. Since no such interactions were observed, it is likely that BG interacts with enzymes involved in the metabolism of the amine.

Abbreviations: PBS, phosphate-buffered saline; FTIR, Fourier transform infrared; HPLC, high-performance liquid chromatography; SCGE, single-cell gel electrophoresis; HA, heterocyclic aromatic amine; BG, β-glucan; BPDE, (+/−)-anti-B[*a*]P-7,8-dihydrodiol-9,10-epoxide; (Trp-P-2), 3-amino-1-methyl-5*H*-pyrido[4,3-*b*]indole; ROS, reactive oxygen species

Introduction

Mushrooms have been used for many years for the preparation of infusions and as foods. Because of their biochemical components, such as carbohydrates, proteins, lipids, and vitamins, they have been considered as functional foods (Muchove, 1989). *Agaricus brasiliensis* S. Wasser

et al., formerly known as *Agaricus blazei* Murril ss. Heinemann, is a mushroom that is native to Brazil. It is used for cooking and also in traditional medicine used for stress, as an immunostimulant, and to improve the quality of life of diabetics. On the basis of several investigations, it has also been proposed that this mushroom has antitumor (Mizuno, 1995), antimutagenic, and anticlastogenic properties (Delmanto et al., 2001; Menoli et al., 2001; Oliveira et al., 2002; Bellini et al., 2003, 2006; Luiz et al., 2003a, b; Guterrez et al., 2004).

Among the polysaccharides found in this mushroom, special attention has been paid to β-glucans (BGs). These polysaccharides have a central linear structure of D-glucose molecules, linked in the β-(1→3) position, containing one or more glucose side-chains (β-(1→6) linkage) (Figure 1). The biological effects of BGs include stimulation of the immune system as well as bactericidal (Tzianabos and Cisneros, 1996) and antiviral (Reynolds et al., 1980) activity. The aim of the present study was to investigate the genotoxic and antigenotoxic effects of BGs in human peripheral lymphocytes. As an endpoint, we used the single-cell gel electrophoresis (SCGE) assay, which is based on the migration of damaged DNA in an electric field (Singh et al., 1988). The potential DNA-protective properties of BGs were studied in combination experiments with Trp-P-2, a heterocyclic aromatic amine (HA) that is formed during the cooking of meats, and with BPDE (the main metabolite of benzo[*a*]pyrene, which is a representative of polycyclic aromatic hydrocarbons).

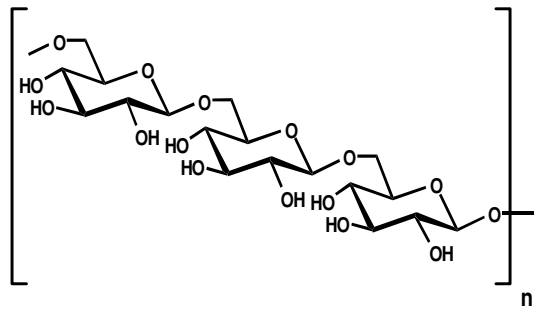

Figure 1. Structure of the β-glucan (1-6) part of the molecule.

To elucidate the potential antioxidant properties of the glucan, additional experiments with hydrogen peroxide (H_2O_2) were conducted. Since it has been shown that polysaccharides such as certain types of fibers inactivate HAs via direct binding (Ferguson et al., 1993; Williams et al., 1999), additional chemical (HPLC) analyses were conducted.

Materials and methods

Extraction of β-glucan from Agaricus brasiliensis

Polysaccharides were prepared from a 5% (w/v) aqueous mushroom suspension, which was heated for 5 h, resulting in an acid medium (pH 5). The material was isolated from the solution after neutralization with 0.1 mol/L NaOH, followed by the addition of 1% (w/v) NaCl (where w is the weight of NaCl and v the volume of the extract) and precipitation in ethanol (1:5 v/v extract–ethanol). The precipitate was separated by centrifugation and clarified with ethanol–hydrogen peroxide solution (1:1 v/v). Owing to partial solubilization of the material, it was subjected to a second extraction using ethanol (4:1 v/v ethanol–clearing medium). The water-soluble portion was lyophilized. Structural characterization by FTIR, ^{13}C NMR and ^{1}H NMR spectroscopy showed a β-glucan–protein complex (Gonzaga et al., 2005b). From this fraction, β-glucan was isolated based on the procedure described by Yoshioka et al. (1985). The suspension was placed on a shaker (15 h) and was centrifuged (8000*g*/60 min). The precipitate was washed with thymol solution in NaCl and dialyzed against water. After successive washings with ethanol (analytical grade), the precipitate was dried in a sand bath (~45°C). The structure of pure β-glucan was confirmed by ^{1}H, 2D-COSY, HMQC and ^{13}C NMR spectroscopy (Gonzaga et al., 2005a).

Chemicals

All chemicals used were of analytical grade. 3-Amino-1-methyl-5H-pyrido[4,3-*b*]indole (Trp-P-2) from Toronto Research Chemicals (Toronto, ONT, Canada) was dissolved in dimethyl sulfoxide (DMSO) from Merck (Darmstadt, Germany). (+/−)-Anti-B[*a*]P-7,8-dihydrodiol-9,10-epoxide (BPDE) from the Biochemisches Institut für Umweltkarzinogene (Grosshansdorf, Germany) was dissolved in DMSO. Hydrogen peroxide (H_2O_2), trypan blue, RPMI medium, and agarose (low- and normal-melting-point agarose) were purchased from Sigma (St. Louis, MO, USA).

Isolation of cells

Blood (10 ml/person) was collected by venipuncture from two donors (one female, one male, both nonsmokers) into heparinized tubes. Lymphocytes were isolated by Ficol centrifugation and subsequently used in genotoxicity tests.

Comet assay

For the comet assays the protocol of Speit and Hartmann (2005) was used. In the genotoxicity experiments, the lymphocytes were exposed to different BG concentrations (20–80 μg/ml) for 40 min. In antigenotoxicity studies, identical concentrations were used and the cells were exposed simultaneously for the same period to Trp-P-2 (150 μmol/L) and for a shorter treatment time (10 min) in experiments with H_2O_2 (50 μmol/L) and BPDE (0.4 μmol/L). The concentrations of the genotoxins were chosen on the basis of earlier experiments. For each experimental point, three cultures were tested in parallel. In all experiments, the viability of the cells was determined with trypan blue (Lindl and Bauer, 1993), and only cultures in which survival was >80 % were evaluated for comet formation.

Afterwards, the cells were washed, centrifuged, and resuspended in culture medium (RPMI). A cell suspension (20 μl, 2×10^4 cells) was mixed with 0.5% low-melting-point agarose (120 μl) at 37°C, and distributed over agarose-coated microscope slides. The slides were covered and kept at 4°C for 20 min. Subsequently, the coverslips were removed and the slides were immersed in lysis solution [89.9 ml lysis buffer (2.5 mol/L NaCl, 100 mmol/L EDTA, 10 mmol/L Tris, pH 10) + 1 ml Triton X-100 + 10 ml DMSO], protected from light, at 4°C, for 1 h. After lysis, the slides were transferred to an electrophoresis chamber and immersed in 2 L buffer solution pH > 13 (300 mmol/L NaOH, 1 mmol/L EDTA, prepared from a stock solution of 10 mol/L NaOH and 200 mmol/L EDTA, pH 10.0) at 4°C for 20 min for denaturation.

Electrophoresis was carried out under standard conditions (25 V, 300 mA, 20 min). Finally, the slides were neutralized with buffer (0.4 mol/L Tris-HCl) for 15 min (3 cycles of 5 min), dried, fixed in 100% ethanol for 10 min, and stored at 4°C until analysis.

Analyses of the microscope slides

The microscope slides were coated with 100 μl of 200 μg/ml ethidium bromide and protected with cover slips. The slides were analyzed using a fluorescence microscope (Nikon model 027012) under 40-fold magnification, with an excitation filter of 420–490 nm and an emission filter of 520 nm. The comet lengths were measured with a computer-aided image analysis system (Helma and Uhl, 2000), and 50 cells were examined for each culture.

Binding assay

The modular liquid chromatograph consisted of two high-pressure pumps (ESA model 420, Chelsmsford, MA, USA), a gradient mixer (Contron model M800, Zurich, Switzerland), and an autosampler (ESA model 465). The analytical column (alpha-chrom C 8 2 × 150 mm,

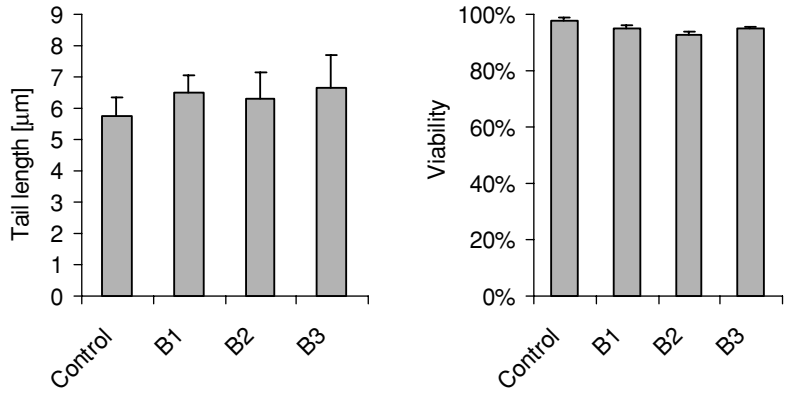

Figure 2. Effect of BG on DNA migration in human peripheral blood lymphocytes. The cells were incubated with the β-glucan for 40 min. Subsequently, the extents of DNA migration (a) and viability (b) were determined. Bars represent means ± SD of results obtained with three slides in parallel.

Upchurch Scientific, Oak Harbour, WA, USA) was thermostated at 30°C. Trp-P-2 was eluted isocratically with a flow rate of 0.6 ml/min. The mobile phase consisted of acetonitrile–methanol–buffer–distilled water (10:15:10:65 by vol). The buffer was prepared by dissolving 3.7 g sodium acetate and 4.9 g trichloroacetic acid in 70 ml water and adding 30 ml glacial acetic acid. Electrochemical detection was carried out with a Coularray electrode system (ESA model 5500). The potentials of the working electrodes were adjusted to +100, +375, +375, +400, +425, +450, +525, and +525 mV. Trp-P-2 was detected in channel 5 (+425 mV).

The binding assay was performed in triplicate by mixing BG (2 mg/ml, pH 7.4) and Trp-P-2 (150 µmol/L) in an Eppendorf tube. Vials were mixed briefly on a vortex mixer and incubated at 25°C on a horizontal shaker table with mild agitation (500 rpm). Parallel controls in triplicate contained 10 µl PBS instead of BG. After 30 min of incubation, adsorption was stopped by mixing 200 µl of the sample with 800 µl mobile phase. For the quantification of Trp-P-2, 20 µl of this solution was injected into the HPLC system. Peak heights were determined with ESA CoularrayWin software.

Statistical analysis

The results of SCGE assays were analyzed by ANOVA, followed by the multiple comparison test of Dunnett, $\alpha = 5\%$. All comparisons were carried out against DMSO (negative control).

Results and discussion

The results of comet experiments in which the genotoxic effects of BG were investigated in human peripheral lymphocytes are summarized in Figure 2. It can be seen that no indication of induction of DNA migration was observed under any condition of test, and also the viability of the cells was not affected. Figure 3 depicts the results of a combined treatment experiment with H_2O_2. The peroxide itself induced a significant increase in DNA migration (i.e., 4-fold over the background); addition of the glucan protected significantly against ROS-induced DNA damage. At the highest dose level tested (80 µl/ml), the extent of DNA damage was reduced by 65%. The findings of experiments with Trp-P-2 and BPDE are shown in Figure 4. Both compounds induced significant induction of DNA migration over the

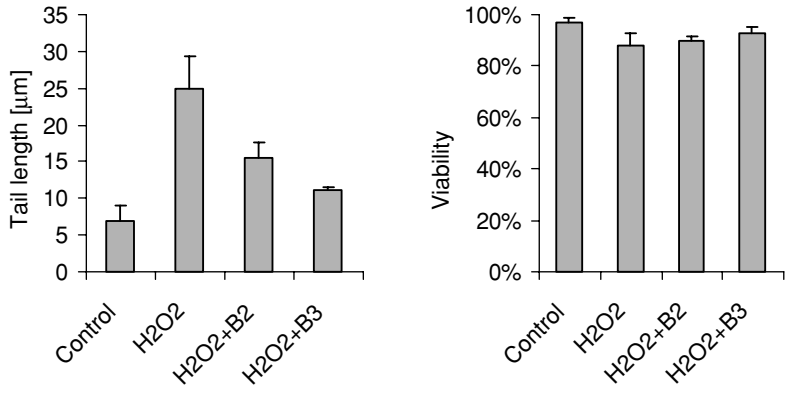

Figure 3. Effect of BG on H_2O_2-induced DNA damage in human peripheral blood lymphocytes. The cells were treated simultaneously with β-glucan (40 and 80 µg/ml) and H_2O_2 (50 µmol/L) for 5 min. Subsequently, the extents of DNA migration (a) and viability (b) were determined. Bars represent means ± SD of results obtained with three slides in parallel.

background level. The glucan had no effect on damage caused by the diol epoxide, but a clear protective effect against the amine was found with concentrations ≥40µl/ml.

Since it is known that certain polysaccharides (e.g., fibers) inactivate HAs via direct binding mechanisms (Schwab et al., 2000; Dashwood, 2002), an additional experiment was carried out in which the amount of free Trp-P-2 was measured by HPLC before and after incubation with BG (49 µl/nl). Since no difference in the adsorption spectrum was observed (Table 1), this can be taken as an indication that binding effects do not account for the prevention of Trp-P-2-induced DNA damage seen in the SCGE experiments.

Taken together, the results of our study show that BG from *Agaricus brasiliensis* is devoid of genotoxic activity and protects human-derived cells against ROS- and Trp-P-2-induced DNA damage, and that there was no reduction in BPDE-induced DNA migration under identical experimental conditions. The protective effects against ROS were not unexpected; Lazarova et al. (2004) also found that fungal β-glucans reduce oxidative DNA damage in in-vitro experiments with rat liver cells. The antioxidant properties of glucans may also account for their antimutagenic effects seen in experiments with cytotoxic drugs (cyclophosphamide, adriamycin, cisplatin) in mice (Tohamy et al., 2003), as it is well known that the mode of action of these antineoplastic compounds involves generation of ROS.

As described above, the prevention of Trp-P-2-induced DNA damage could not be explained by direct binding effects. It is conceivable that the mode of action of BG involves alterations of the activities of enzymes that catalyze activation/detoxification reactions. In this context, it is notable that Japanese groups (Hashimoto et al., 2002; Okamoto et al., 2004) showed recently that fungus-derived polysaccharides inhibit cytochrome P450 1A isoenzymes, including CYP1A2 which catalyzes the first activation step of HAs (*N*-hydroxylation). Since no protective

Table 1. Effect of the combination of Trp-p2 and BG on absorption spectrum

	Peak height (nA)	Mean ± SD (nA)
Trp-P-2	685	
	693	689 ± 4
	690	
Trp-P-2 + β-glucan	685	
	692	687 ± 4
	684	

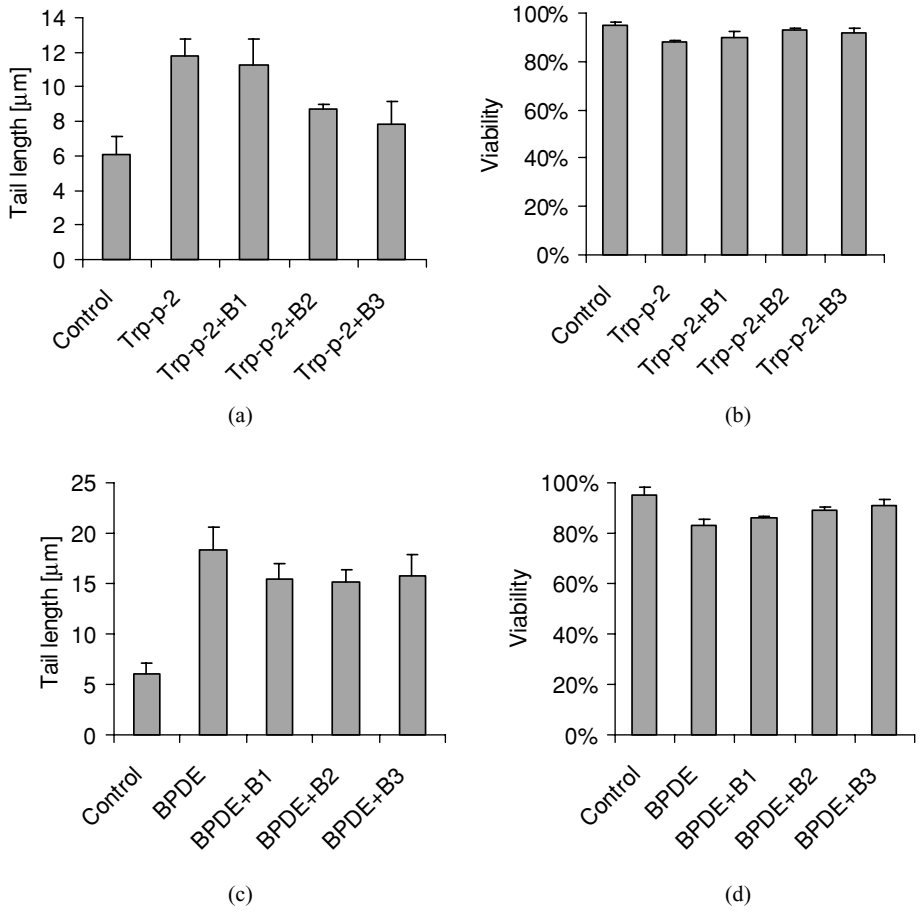

Figure 4. Effect of BG on Trp-P-2- and BPDE-induced DNA migration in human peripheral blood lymphocytes. The cells were exposed to Trp-P-2 or BDPE for 40 min in the presence of defined amounts of BG. Subsequently, the extents of DNA migration (a, c) and viability (b, d) were determined. Bars represent means ± SD of results obtained with three slides in parallel.

effects were seen in the combination experiments with BPDE (Figure 4a), it can be excluded that the protective effects involve the induction of glutathione *S*-transferases, involved in the detoxification of diol epoxides (Steinkellner et al., 2001) and also electrophilic metabolites of HAs. Therefore, the most probable assumption is that BG inhibits phase I enzymes involved in the activation of HAs and other procarcinogens. Since our results show that protective effects take place in human-derived cells, it can be tentatively assumed that consumption of *Agaricus brasiliensis* may also protect humans against reactive oxygen species and HAs.

Acknowledgments

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