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Action of bioactive compounds in cellular oxidative response

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Abstract

Nowadays there is a growing demand for biodiesel production which can be made, among other resources, from mushroom biomass. It is well established the continuous growth of the population affected with premature aging and numerous diseases in which reactive oxygen species (ROS) are implicated. This fact leads to an increasing consumption of antioxidant rich nutrients, such as mushrooms, which are effectively produced on lignocellulosic biomass substrate. The aim of this study was to investigate, in brain slices, the effect of polysaccharides extracted from the *Boletus edulis* and *Tricholoma equestre* mushroom species, in the formation of neuronal ROS, using the fluorescent indicator H_2DCFDA. The polysaccharides compounds from both mushroom species had no effect on ROS signals at 0.1 g/L and 0.5 g/L, but decreased the amplitude of the signal at the concentration of 1.0 g/L. These results are in agreement with the idea that polysaccharides have antioxidant properties. Besides the nutritional value of mushrooms, the biomass residues from their production can be used as raw material for bioethanol and biobutanol production, being the later an even more efficient biofuel than ethanol or methanol.

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0. Introduction

In addition to the highly important nutritional and medical mushrooms properties, their wastes, such as substrate and even mushrooms under improper conditions can be used as biomass. This is a very promising renewable resource in energy production, as happens with all waste with energy potential that may be used for this purpose [1].

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A high number of pathologies, including cancer and neurodegenerative conditions are caused by oxidative damage, due to the action of free radicals in the body [2]. For this reason, it becomes increasingly necessary and important the consumption of products with antioxidant properties. Thus, considering the restrictions on the use of synthetic antioxidants, it is now common to search for natural products, which are easily degradable and may reduce the negative oxidative cellular effects caused by free radicals such as reactive oxygen species (ROS) [3,4].

Among the large number of natural products, mushrooms have been the target of numerous studies, due to their high nutritional properties and beneficial effects on health. These fungi are quite rich in bioactive compounds, such as polysaccharides, phenolic compounds, vitamins and secondary metabolites, containing high antioxidant properties [3,5,6].

Reactive oxygen species are formed by living organisms during normal cellular activity and are also triggered by a variety of agents including ultraviolet light, ionizing radiation, therapeutic drugs and inhaled air contaminants [3,7].

During normal metabolism, ROS are formed and eliminated from the organism in a balanced way but, following strong stimulation, a large increase in ROS production may occur causing oxidative stress. In excess ROS may cause serious cellular structural damage in lipids, proteins and DNA, being thus implicated in a variety of pathologies, such as neurodegenerative ones, cancer, hypertension and diabetes [8]. Biological ROS detection can be made using different fluorescent probes including H_2DCFDA, an organic compound that is a reduced form of fluorescein, which reacts with hydrogen peroxide and hydroxyl radicals [9]. This indicator is a non-fluorescent reduced form of fluorescein, which following cleavage by intracellular esterases becomes a bright green fluorescent compound, 2′ ,7′ - dichlorofluorescein (DCF).

The brain, which has a large fat content, namely of polyunsaturated fatty acids, is highly sensitive to oxidative stress since it consumes much O_2 and produces a lot of free radicals that may be harmful. For these reasons it is one of the major targets of free radicals, which may "tear" the cell membranes and alter neural communication and DNA [10].

Antioxidants, which are aromatic compounds containing one or more hydroxyl groups, form the main defense mechanism that protects organisms from excessive ROS formation. Enzymatic antioxidants (superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) form the first-line defense against ROS by interacting directly with oxidizing cellular compounds [10]. Additional protection is obtained from food nutrients such as polyphenols, flavonoids, carotenoids, polysaccharides, terpenes, vitamins C and E and essential minerals including zinc, selenium, iron and manganese [11].

We have previously found that polysaccharide extracts of the mushrooms *Boletus edulis* and *Tricholoma equestre* contain a diversity of sugars, being the most abundant galactose, which accounts for about 50% of the total sugar content. Other sugars present in both mushrooms in significant amounts are glucose and fructose. The *Boletus edulis* mushrooms have yet additional sugars namely, mannose, glucosamine and ribose, in small amounts.

The goal of this work was to evaluate the action of polysaccharides extracts from two mushroom species, *Boletus edulis* (BE) and *Tricholoma equestre* (TE) in the production of neuronal ROS. The results, obtained from brain slices using the fluorescent ROS indicator H_2DCFDA, indicate that at the higher concentration (1.0 g/L) the ROS signals are depressed, consistent with an antioxidant neuronal role.

Residues of mushrooms extracts have already been used as biomass to produce biobutanol, which is a biofuel with a behavior similar to that of gasoline in the combustion process, but with the advantage of being non-polluting [1,12,13]. Recently it was found that there is a bacterium (TG57, *Thermoanaerobacterium thermosaccharolyticum*), which has the ability to convert cellulose to biobutanol. This bacterium is widely found in the biomass remains of mushroom production [14]. The great advantage is that this biofuel can be used directly in gasoline engines, unlike other alternative fuels [1,12,13]). More studied and already in use in some sectors is bioethanol. This biofuel is a natural alcohol produced by fermenting sugar extracted from sugary plants and by enzymatic hydrolysis of starch contained in some cereals. These sugars can also be obtained through fungi, requiring a shorter pretreatment time while remaining rich in cellulose and hemicellulose [15,16]. Biofuels can also be used in fireplaces (bio-fireplaces) and burners, since they have a 50% higher calorific value than that of wood [1,12,13].

1. Material and methods

The experiments were performed in brain slices from female Wistar rats, with ages between 8 and 16 weeks and with 16 to 18 days of pregnancy. The data were obtained from slices (400 μm thick) of the hippocampus, at the

CA3 area that contains the mossy fibers synaptic system, playing the CA3 region a key role in memory formation and in neurodegeneration [17].

All efforts have been made to minimize animal suffering and to reduce the number of animals needed to produce reliable scientific data. The animals were sacrificed by cervical dislocation.

The slices were kept in an oxygenated (with 95% O_2 and 5% CO_2) artificial cerebrospinal fluid (ACSF), with pH 7.4, at room temperature. This solution, which reproduces the extracellular environment, was prepared with ultrapure water (18.2 MΩ cm) (Interlab Direct — Pure system) adding the solute compounds in the following composition (in mM): NaCl 124, $NaHCO_3$ 24, KCl 3.5, $MgCl_2$ 2, NaH_2PO_4 1.25, $CaCl_2$ 2 and D-Glucose 10. In the experimental chamber the slices were perfused at 1.5–2.0 mL/min, with an oxygenated medium (ACSF without or with polysaccharides) at 32 °C. Three polysaccharides solutions, with concentrations 0.1, 0.5 and 1.0 g/L, were prepared from extracts of the mushroom species *Boletus edulis* or *Tricholoma equestre*.

The polysaccharide extracts were ground with the aid of a mortar, to reduce the particle size and then added to the ACSF solution and homogenized, being the resulting solution filtered afterwards. The ROS signals were obtained from slices incubated, for one hour, in an ACSF oxygenated solution containing the permeant fluorescent ROS indicator, 2′, 7′- dichlorofluorescin diacetate (H_2DCFDA) (20 μM).

After the incubation, the slices were perfused with ACSF at 32 °C at a rate of 1.5 to 2.0 mL/min in an experimental chamber mounted in a fluorescence microscope (Zeiss Axioskop) equipped with a water immersion objective (40x, N.A. 0.75, 1.6 mm working distance).

Transfluorescence optical signals were recorded at the mossy fiber synaptic system from CA3 hippocampal area, using an halogen light source (12 V, 100 W), an arrow band (10 nm) excitation and a high pass emission filters of 480 nm and 500 nm, respectively, and a silicon photodiode (Hamamatsu K2G 1336, 1.0 mm^2). The data were acquired with a sampling frequency of 0.016 Hz and processed using a 16 bit data acquisition system and the Signal Express analysis software from National Instruments. The optical signals, corrected for constant autofluorescence, represent the ratio of the total fluorescence (F_T) over baseline fluorescence (F_0) obtained as the average of the first 10 points. Data represent the mean ± S.E.M. Statistical significance was assessed by means of the Mann–Whitney U test, $p < 0.05$ was considered significant.

Chemicals used were: NaCl (José Manuel Gomes dos Santos, Lda, Odivelas, Portugal) (purity ≥ 99.7%), $MgCl_2$ (Merck, Lisbon, Portugal), $NaHCO_3$ (purity ≥ 99.7%), KCl (purity ≥ 99.0%), NaH_2PO_4 (purity ≥ 99.0%), $CaCl_2$ (purity ≥ 99.0%), D-Glucose (purity ≥ 99.5%) (Sigma-Aldrich, Sintra, Portugal) and H_2DCFDA (Invitrogen by Thermo Fisher Scientific, USA). The polysaccharides were extracted from the wild mushroom species *Boletus edulis* collected in Alfândega da Fé, Bragança, Portugal and *Tricholoma equestre* collected at a forest in the region of Cantanhede, Portugal. The residues of these mushrooms have high added value if used as biomass for clean energy generation.

2. Results and discussion

The optical signals were collected at the hippocampal mossy fiber synapses from CA3 area. The effects induced by mushroom polysaccharides extracts on the ROS signals, recorded with the fluorescent indicator H_2DCFDA, are shown in the next figures. In all experiments the slices were perfused initially with ACSF, for 10 min, then with the polysaccharides containing medium during 30 min and afterwards again with ACSF, also for a 30 min period.

As shown in Fig. 1 the smaller concentrations of the polysaccharides extracted from *Boletus edulis* induce small decreases in the signals. At 0.1 g/L (Fig. 1a) there is a reduction of about 2.2% in the period 35–40 min (n = 2), recovering the signal to 0.7% below the baseline, following washout. In the presence of the concentration of 0.5 g/L polysaccharides (Fig. 1b), the ROS signals became 2.6% smaller at 35–40 min (n = 2) than the control value. Upon ACSF reperfusion the intensity increased to baseline values, showing an enhancement of 0.9%. Larger ROS changes were observed for the concentration of 1.0 g/L (Fig. 1c), namely a depression measuring 18.2% of control at 35–40 min. Upon returning to the ACSF solution, the ROS signals increased above the baseline having an amplitude of 6.2% at 65–70 min with respect to the control level.

Fig. 2 shows again the effect on neuronal ROS signals of the same three concentrations (0.1, 0.5 and 1.0 g/L) of polysaccharides extracted, in this case, from *Tricholoma equestre* mushrooms.

It can be observed (Fig. 2a) that the application of the lower concentration, 0.1 g/L, did not cause significant changes with respect to the baseline or during the last 30 min (n = 3), following the compound removal. Similar results, i.e. no clear changes, were obtained with the intermediate concentration of 0.5 g/L (n = 3), but there was

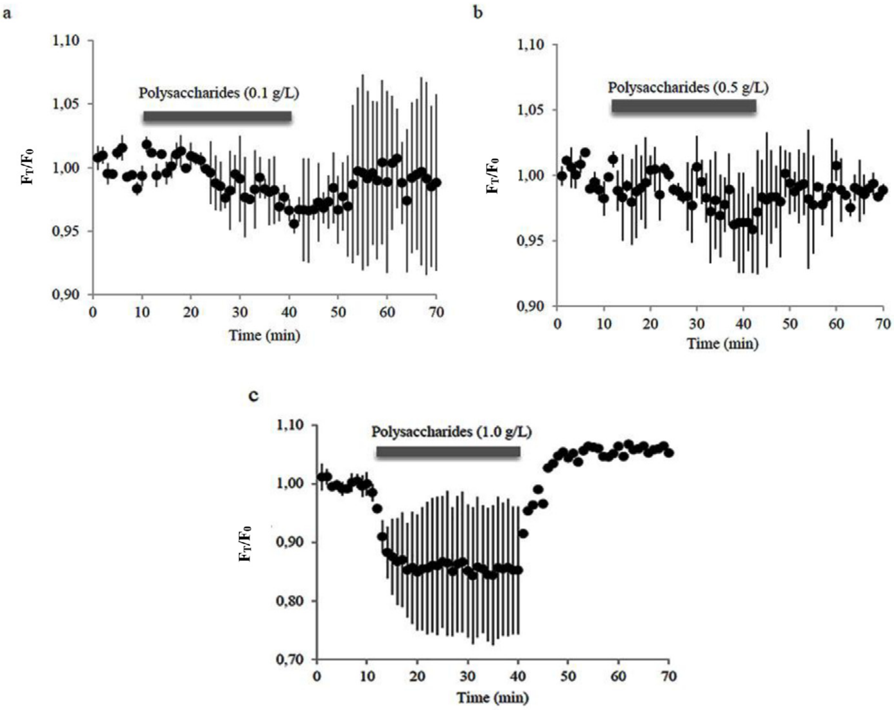

Fig. 1. Neuronal ROS changes induced by the polysaccharides extracted from the *Boletus edulis* mushroom. The signals were obtained at the concentrations of (a) 0.1 g/L (n = 2), (b) 0.5 g/L (n = 2), (c) 1.0 g/L (n = 4 from $t = 0$ to $t = 40$ min and n = 2 after $t = 40$ min). The bars represent the period of application of the polysaccharides solutions. All values were normalized by the average of the first 10 responses and represent the mean ± S.E.M.; F_T, total fluorescence; F_0, basal fluorescence.

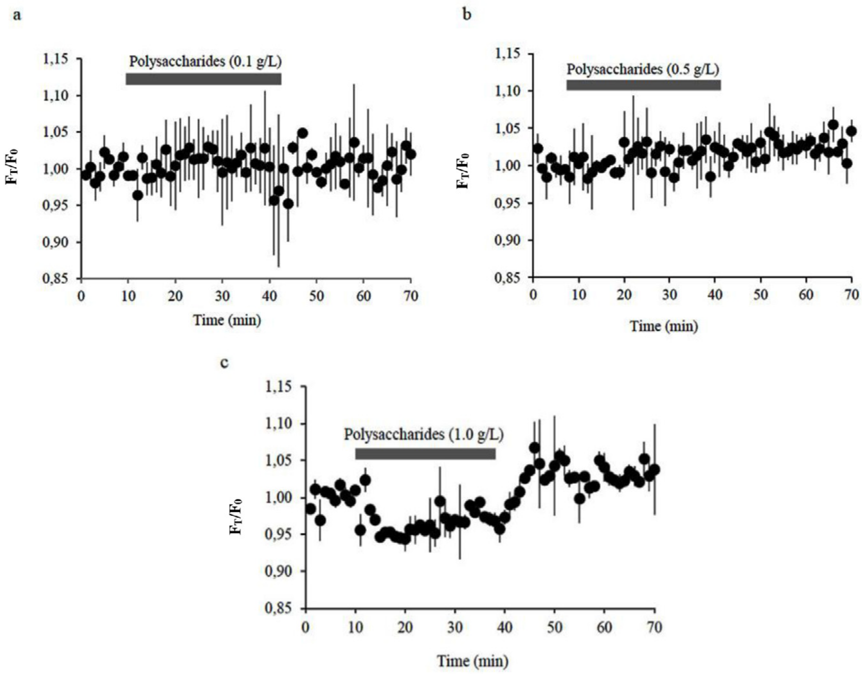

Fig. 2. ROS signals evoked by the polysaccharides extracted from the *Tricholoma equestre* mushroom. The extracts were applied at the concentrations of (a) 0.1 g/L (n = 3), (b) 0.5 g/L (n = 3), (c) 1.0 g/L (n = 3) during the period indicated by the bars. The data, normalized by the average of the first 10 points, indicate the mean ± S.E.M.; F_T, total fluorescence; F_0, basal fluorescence.

a small increase of the signal at the end of the washout period, although the concentration is five times higher than the first one (Fig. 2b). Applying an even higher concentration, 1.0 g/L, led to the observation of ROS changes that were reduced with respect to baseline, by 3% (n = 3) in the period 35–40 min (Fig. 2c). This result indicates that the polysaccharides solution had an impact in the amount of ROS formed, leading to its reduction, thus attenuating their possible negative effects. In the final 30 min, during the ACSF reperfusion, the signals recovered and attained an enhanced level indicating that the polysaccharides effect was reversible.

To compare the amplitudes of the changes induced by the different types and concentrations of polysaccharides, a bar graph is represented in Fig. 3, where the amplitude of the bars indicates the average value of the final 5 min of the signal, in the corresponding media. As can be seen in this figure, the two types of polysaccharides, extracted from *Tricholoma equestre* or from *Boletus edulis* mushrooms, have no significant effect on ROS signals at the lower concentration, 0.1 g/L. However, at 0.5 g/L, the signals decreased slightly in the presence of the *Boletus edulis* extract but did not change for the *Tricholoma equestre* one. For the higher concentration, 1.0 g/L, both ROS changes were depressed.

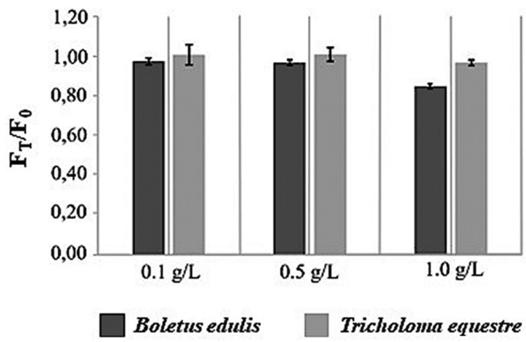

Fig. 3. Normalized amplitude of ROS signals evoked by different concentrations of the polysaccharides extracts. The amplitude of the bars represents the average ± S.E.M. of the last five data points obtained in each medium. F_T, total fluorescence; F_0, basal fluorescence.

Mushrooms have a number of advantages, both for their antioxidant capacity and for their sustainable benefits. Their waste is a source of biomass used to produce biofuel, for motors and heating.

3. Conclusions

This study shows some of the potential that mushrooms have on health and refers to positive effects that the use of their biomass may have on the environment.

For the lower and middle polysaccharides concentrations, during the ACSF reperfusion, the signals remained at or returned to the baseline or to a level very close to it, indicating that the polysaccharides effect, if it existed, was reversible. A different situation occurred for the higher concentration which caused a reversible depression.

The results suggest that, at certain concentrations, the applied polysaccharides inhibit the production of neuronal ROS, thus contributing to the reduction of their possible negative effects. These facts support the idea that polysaccharides have antioxidant properties acting as biological response modifiers, indicating that, as expected, the polysaccharides lower the amount of these radicals.

The use of mushrooms biomass to produce cellulosic biofuels is growing due to concerns about sustainable energy and low greenhouse gas emissions in the future. Cellulosic butanol has already been used, however its production is still hampered by the lack of more bacteria capable of converting cellulosic biomass into biofuels. Bioethanol is used on a larger scale and is obtained by safe burning without the release of odors or residues. Currently, this product is one of the main sources of renewable energy, since it is 100% ecological and does not contribute to the global warming of the planet.

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