Carbohydrate Polymers 197 (2018) 598–607

Contents lists available at ScienceDirect

Carbohydrate Polymers

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

Neuroprotective properties of *Cantharellus cibarius* polysaccharide fractions in different *in vitro* models of neurodegeneration

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ARTICLE INFO

Keywords:
Cantharellus cibarius
Golden chanterelle
Neurons
Excitotoxicity
Trophic stress
Antioxidant

ABSTRACT

The percentage of people suffering from neurodegenerative diseases is constantly increasing, because of that searching for substances able to prevent or inhibit neuronal death sseems to be reasonable. Because of the high popularity the search of new neuroprotective agents we started from *Cantharellus cibarius*. Neuroprotective properties of *C. cibarius* polysaccharides fractions was investigated in different models of neurodegeneration including trophic stress, excitotoxicity and andoxidative stress. Fractions influence on neurons viability was examined using Neurite Outgrowth Staining, MTT and LDH tests, while antioxidant capacity was determined by commercial antioxidant assays. Performed studies revealed beneficial effect of *C. cibarius* fractions (CC2a, CC3) on neurons viability and neurite outgrowth in normal and different stress conditions. Both tested fractions have shown antioxidant capacity and effectively neutralize the negative changes induced by glutamatergic system activators. Discovered neuroprotective properties of investigated compounds suggested the their use for developing effective and safety therapeutic strategy for neurodegenerative diseases.

1. Introduction

Despite the progress that has been made in neuroscience in recent years, the percentage of people suffering from neurodegenerative diseases like amyotrophic lateral sclerosis, Alzheimer's, Huntington's, Parkinson's is constantly increasing (Brown, Lockwood, & Sonawane, 2005). Due to the insufficient effectiveness of the available therapeutic strategies, high hopes are placed in neuroprotection defined as prevention of neuronal structure and/or function from various damaging factors using substances capable of preventing or inhibiting neuronal death (Brown et al., 2005; Vajda, 2002).

Due to the wide spectrum of biological activities and the large therapeutic potential of polysaccharides isolated from *Basidiomycetes* (Devi & Maiti, 2016; Lemieszek & Rzeski, 2012; Wasser, 2002; Zaidman, Yassin, Mahajna, & Wasser, 2005) we decided to take a look at this group of compounds aiming to discover effective and safe neuroprotective agents. So far neuroprotective abilities have been shown in case of polysaccharides isolated from *Agaricus bisporus* (Amal, Mona, & Rasha, 2015), *Cordyceps cicadae* (Olatunji et al., 2016), *Cordyceps militaris* (Tsai, Yen, & Yang, 2015), *Ganoderma lucidum* (Aguirre-Moreno et al., 2013; Gokce et al., 2015; Lai et al., 2008; Tello et al., 2013; Zhang et al., 2011; Zhao, Lin, Liu, & Lin, 2004; Zhou et al., 2010), *Hericium erinaceus* (Cheng, Tsai, Lien, Lee, & Sheu, 2016; Han, Ye, & Wang, 2013; Park et al., 2002; Wong, Kanagasabapathy, Bakar, Phan, & Sabaratnam, 2015), *Innonotus obliquus* (Yue, Xiuhong, Shuyan, & Zhonghua, 2015), *Lignosus rhinocerotis* (Seow et al., 2015), *Sparassis crispa* (Hu et al., 2016), *Tremella fuciformis* (Jin, Hu, Zhang, & Liu, 2016; Kim et al., 2007). Despite the limited evidences, which proved neuroprotective effect of mushrooms polysaccharides, available data indicate such potential and provide incentives for further research.

Because of the high popularity both in the terms of its occurrence (cosmopolitan species) as well as consumption (species prized for its taste and smell) the search of new neuroprotective agents we started from *Cantharellus cibarius* (golden chanterelle). Furthermore, recent reports which gradually discover beneficial properties of *C. cibarius* including anticancer, anti-inflammatory, immunomodulatory, antigenotoxic, antiaging and antioxidant (Drewnowska & Falandysz, 2015; Ebrahimzadeh, Nabavi, Nabavi, & Eslami, 2010; Ebrahimzadeh,

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https://doi.org/10.1016/j.carbpol.2018.06.038
Received 4 February 2018; Received in revised form 10 April 2018; Accepted 7 June 2018
Available online 08 June 2018
0144-8617/

Safdari, & Khalili, 2015; Hong et al., 2012; Muszyńska, Sułkowska-Ziaja, & Ekiert, 2011; Muszyńska, Kała, Firlej, & Sułkowska-Ziaja, 2016; Palacios et al., 2011; Valentão et al., 2005) attracted our attention to bioactive compounds presented in golden chanterelle as well as pointed out the need for further investigation to broaden the therapeutic applications of this mushroom.

The main aim of the presented study was the evaluation the neuroprotective activity of polysaccharide fractions isolated from *C. cibarius* in different *in vitro* models of neurodegeneration, including trophic stress, excitotoxicity and oxidative stress. Studies were conducted in neurons derived from human neuroblastoma cell line SH-SY5Y.

2. Materials and methods

2.1. Reagents

All reagents and kits were purchased from Sigma-Aldrich (St Louis, MO, USA) unless otherwise indicated. Stock solutions of polysaccharide fractions (10 mg/ml) were prepared in PBS (buffered saline solution) and stored at 4 °C. Working solutions (10, 25, 50, 100 μg/ml) were prepared by dissolving an appropriate stock solution in culture medium.

2.2. Fungal origin

The wild-grown fruiting bodies of *Cantharellus cibarius* were collected in a chestnut orchard (Macedo de Cavaleiros 41°35' N and 6°57' W, 700 m altitude) and in a mixed forest stand of pine and oak (Vila Real (41°19' N and 7°44' W, 479 m altitude). Representative voucher samples of the wild-gathered species were deposited at the mycological herbarium of the University of Trás-os-Montes e Alto Douro.

2.3. Extraction and purification of Cantharellus cibarius fractions

For the preparation of the AIR (Alcohol Insoluble Residue), *Cantharellus cibarius* mushrooms were freeze-dried and ground to a fine powder. To 700 ml of 80% ethanol at 80 °C 100 g of mushroom powder were added, and the suspension was boiled with stirring during 1 h. After this time, the insoluble material was filtered through glass fiber and washed twice with 50 ml of 80% ethanol. The AIR was dried in a forced air oven at 50 °C during 12 h. For the preparation of the water soluble material (WSM), to 700 ml of boiling water, it was added 100 g of the AIR, and the suspension was boiled with stirring during 1 h. After this time, the insoluble material was separated from the supernatant by centrifugation (6000 rpm, 20 min, 4 °C). The water insoluble material was washed with water and separated again by centrifugation as before. The supernatant were combined, dialyzed (MW cut off 12–14 kDa) against water (6 water renewals) and freeze dried yielding the *Cantharellus cibarius* yielding the water soluble biopolymers (WSB). The *Cantharellus cibarius* WSB, including polysaccharide and glycoproteins present in the WSM and isolated by dialysis were further purified by fractionation by anion exchange chromatography on a Q-Sepharose FF stationary phase (loaded on a XK16/20 column, Pharmacia). The eluent was a pH 4.5 Na-acetate 5 mM buffer containing 0.02% sodium aside. WSB (at a concentration of 1 mg/ml) was applied to the column and, after application, the column was flushed with a minimum of 4 column volumes of the initial buffer or until the absorbance at 280 nm reached the initial level. The retained material was eluted by gradient elution, 0 to 500 mM NaCl in 5 h, 500 mM to 1000 mM in 3 h and 1000 mM to 2000 mM in 2 h. Fractions (2 ml) were collected and assayed for sugars by the phenol–sulfuric acid method and continuously monitored at 280 nm. The appropriated fractions were polled, dialysed (12–14 kDa cut-off), and freeze-dried. Four fractions were obtained one not retained on the anion-exchange column CC1 and three retained on the anion exchange column and eluted with increasing salt concentrations CC2a, CC2b, CC3. Sugar composition of the AIR, WSM, WSB and purified fractions were determined after hydrolysis with sulfuric acid 1 M at 100 °C during 2.5 h, using between 2 to 5 mg of sample. After hydrolysis 0.5 ml of internal standard was added (2-deoxyglucose at 1 mg/ml), and the solution was diluted 10 times before analysis by high performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD, ICS-3000, Dionex). The separation was performed with a CarboPac PA-20 column (150 mm × 3 mm) with a CarboPac PA20 pre-column (Dionex) using eluent A – 1.25 mM NaOH solution containing 2 mM $Ba(OH)_2$, eluent B – 400 mM sodium acetate containing 2 mM $Ba(OH)_2$ and eluent C – 500 mM NaOH containing 2 mM $Ba(OH)_2$. The eluent was kept under nitrogen all times to reduce carbonate buildup and biological contamination. The injection volume was 5 μl, the flow rate was 0.3 ml/min and the column temperature was maintained at 35 °C during the run. The following elution program was used: 0–19 min, 100% A, increase to 50% B until 27 min and maintained until 37 min; increase to 40% C and decreasing to 0% B until 47 min and maintained until 57 min. The column was conditioned with 100% A during 15 min before injection. Electrochemical detector consisted of Au working electrode, Ag/AgCl reference electrode, and Ti counter electrode. The ED cell waveform was +0.1 V from 0.00 to 0.40 s, then –2.0 V from 0.41 to 0.42 s, and a ramp –2.0 to +0.6 V from 0.42 to 0.43 s, followed by –0.1 V from 0.44 to 0.50 s (end of cycle). The integration region was from 0.2 s to 0.4 s.

2.4. Size exclusion chromatography

Size exclusion chromatography on Sephacryl S-400 HR was performed on a column width of 2.6 and 70 cm length (XK 26/70, Pharmacia) at a flow rate of 0.3 ml/min. Samples (2 mg) were suspended in 1 ml of 100 mM Na phosphate buffer, pH 6.5, containing 3 M urea. The same phosphate/urea buffer was used as the eluent. Fractions (1 ml) were collected and assayed for sugars with the phenol–H_2SO_4 method, and the eluent was continuously monitored at 280 nm. Exclusion and total volumes were calibrated with Blue Dextran and Glc, respectively.

2.5. Methylation analysis

Polysaccharides were activated with powdered NaOH and methylated with CH_3I (Ciucanu & Kerek, 1984; Isogai, Ishizu, & Nakano, 1985) as described by Coimbra, Delgadillo, Waldron, and Selvendran (1996), followed by a remethylation to ensure complete methylation of the polysaccharides (Nunes & Coimbra, 2001). The remethylated material was hydrolyzed with 2 M trifluoroacetic acid (Harris, Henry, Blackeney, & Stone, 1984), and the partially methylated sugars were reduced with NaBD4 and acetylated with acetic anhydride with 1-methylimidazole as catalyst. The partially methylated alditol acetates (PMAAs) were identified by gas chromatography-mass spectrometry (Nunes & Coimbra, 2001) and quantified by gas chromatography-flame ionization detection using the molar response factors of Sweet, Shapiro, and Albersheim (1975).

2.6. Cell line

Human undifferentiated neuroblastoma cell line SH-SY5Y was purchased from ECACC (European Collection of Cell Cultures, Salisbury, UK.). Cells were grown in 1:1 mixture of Ham's F12 nutrient and Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 1% non-essential amino acid solution, penicillin (100 U/ml) and streptomycin (100 mg/ml). Cells were maintained in a humidified atmosphere of 95% air and 5% CO2 at 37 °C (standard conditions).

2.6.1. Differentiation of SH-SY5Y towards neuronal cells

For induction of the neuronal differentiation, SH-SY5Y cells at a