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Polysaccharides purified from *Cordyceps cicadae* protects PC12 cells against glutamate-induced oxidative damage

Opeyemi J. Olatunji[a,1], Yan Feng[a,1], Oyenike O. Olatunji[b], Jian Tang[a], Yuan Wei[a], Zhen Ouyang[a,*], Zhaoliang Su[c]

[a] School of Pharmacy, Jiangsu University, Zhenjiang, 212013, China
[b] Department of Pharmacognosy and Pharmaceutical Botany, Faculty of Pharmaceutical Sciences Prince of Songkla University, Hat Yai, 90112, Thailand
[c] School of Medical Science and Laboratory Medicine, Jiangsu University, Zhenjiang, 212013, China

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ABSTRACT

Two polysaccharides CPA-1 and CPB-2 were isolated purified from *Cordyceps cicadae* by hot water extraction, ethanol precipitation and purification using anion exchange and gel filtration chromatography. Preliminary structural characterization of CPA-1 and CPB-2 were performed. The protective effect of CPA-1 and CPB-2 against glutamate-induced oxidative toxicity in PC12 cells was analyzed. The results indicated that pretreatment of PC12 cells with CPA-1 and CPB-2 significantly increased cell survival, Ca^{2+} overload and ROS generation. CPA-1 and CPB-2 also markedly up-regulated the antioxidant status of pretreated PC12 cells. Our results suggested that *Cordyceps cicadae* polysaccharides can protect PC12 cells against glutamate excitotoxicity and might serve as therapeutic agents for neuronal disorders.

1. Introduction

Polysaccharides belong to a class of polymeric carbohydrate consisting of monosaccharides that are connected through glycosidic bonds as branched or unbranched chains (Zong, Cao, & Wang, 2012). Fungal-derived polysaccharides have gained popularity in recent years due to the wide range of chemical diversity and bioactivities they display, including antioxidant, immunomodulatory, hepatoprotective, anti-inflammatory, antitumor, steroidogenic, hypolipidemic and hypocholesterolemic properties (Cheung, 1996; Cheung et al., 2009; Fisher & Yang, 2002; Gondim et al., 2012; Muller et al., 1996; Rashid et al., 2011; Sun et al., 2009; Wu et al., 2012; Yu et al., 2004; Zhang et al., 2012; Zhang, Cui, Cheung, & Wang, 2007).

Oxidative stress plays an important role in several neuronal disorders such as Parkinson's disease (PD) and Alzheimer's disease (AD) (Halliwell, 2006), and it occurs when there is an imbalance between the oxidative and antioxidant system within the cell leading to an increase in the generation of reactive oxygen species (ROS) and peroxyl radicals. Increase in the level of ROS causes damages to the DNA, proteins and mitochondrial of cells (Surendran & Rajasankar, 2010). Therefore, the use of antioxidants to eradicate ROS may be an effective means in the treatment of neurodegenerative diseases (Soto-Otero, Méndez-Alvarez, Hermida-Ameijeiras, Muñoz-Patiño, & Labandeira-Garcia, 2000).

Glutamate is a neurotransmitter mediating excitatory synaptic responses. It plays a pivotal role during the development of neurons through the activation of glutamic receptors present in the central nervous system (Jin, Horning, Mayer, & Gouaux, 2002; Molnar & Saac, 2002). However, excessive quantities of extracellular glutamate have been implicated in the pathogenesis of neuronal disorders (Tokarski, Bobula, Wabno, & Hess, 2008; Tzschentke, 2002). Two main mechanisms are involved in glutamate neurotoxicity; oxidative stress and glutamate receptor-mediated neurotoxicity. The first one is associated with glutamate-induced oxidative stress as a result of the prevention of cystine uptake. The reduced uptake of cystine, a precursor of glutathione leads to a decrease in the antioxidant defense resulting in oxidative stress (Albrecht et al., 2010; Pereira & Oliveira, 2000). Neuronal cell death induced by oxidative stress is mediated through the production of reactive oxygen species (ROS), mitochondrial dysfunction and the stimulation of several apoptosis-related deaths signaling pathways (Penugonda, Mare, Goldstein, Banks, & Ercal, 2005). The glutamate

* Corresponding author at: School of Pharmacy, Jiangsu University 301 Xuefu road, Zhenjiang, 212013, China.
E-mail address: zhenouyang@ujs.edu.cn (Z. Ouyang).
[1] These two authors contributed equally to the work.

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receptor mediated excitotoxicity occurs through the activation of glutamate receptors (NMDA receptors), leading to the massive influx of Ca^{2+} and cell death (Lipton & Rosenberg, 1994; Michaels & Rothman, 1990). High concentrations of glutamate can lead to PC12 cell toxicity, and antioxidants have been proven to show protective effects against the cytotoxicity induced by glutamate in PC12 cells (Kawakami, Kanno, Ikarashi, & Kase, 2011; Li, Liu, Dluzen, & Jin, 2007; Penugonda et al., 2005). The exposure of PC12 cells to lofty concentrations of glutamate can be used as a simple but efficient experimental model for studying the neuroprotective effects as well as exploring the molecular mechanistic pathways involved.

Cordyceps cicadae is an entomopathogenic fungi which belongs to the family Clavicipitaceae in the order Hypocreales (Tang & Eisenbrand, 1992). It is a well-known traditional Chinese medicine which has been in use for thousands of years as treatment for fatigue, night sweat, fever, infantile convulsion, palpitation and dizziness (Kuo et al., 2002). Previous researchers have demonstrated that polysaccharides isolated from *Cordyceps cicadae* displayed potent pharmacological properties such as immunomodulatory, renal protective, anti-oxidative properties (Kim et al., 2011, 2012; Ren, He, Cheng, & Chang, 2014). Recently, pilot studies conducted in our laboratory indicated that the crude polysaccharide extracts from *Cordyceps cicadae* displayed antioxidant and neuroprotective effects. Therefore, in this present study, we report the isolation, purification as well as the neuroprotective activity of two biological polysaccharides obtained from *Cordyceps cicadae*.

2. Materials and methods

2.1. Chemical and reagent

Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were obtained from Gibco Industries Inc. (Grand Island, NY, USA). 3-(4,5-dimethylthiazol-2-yl)- 2,5-diphenyltetrazolium bromide (MTT), L-glutamate, and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich (Missouri, USA). DCFH-DA ROS assay kit was purchased from Beyotime Institute of Biotechnology (Jiangsu, China). Lactase dehydrogenase (LDH), glutathione (GSH-Px), superoxide dismutase (SOD), malondialdehyde (MDA) assay kits were procured from NanJing Jiancheng Bioengineering Institute (Jiangsu, China). DEAE-52 cellulose and Sephadex G-100 were purchased from GE Healthcare (Waukesha, WI, USA). All other chemicals and reagents used were of analytical grade. PC12 cells were obtained from the Institute of Medical Science and Laboratory Medicine, Jiangsu University, Zhenjiang, China.

2.2. Plant material

Cordyceps cicadae were collected from Jurong, Jiangsu Province, China and authenticated by Professor Zhen Ouyang. A voucher specimen (CC-0015) was deposited in the herbarium of the School of Pharmacy, Jiangsu University.

2.3. Extraction and isolation

Dried powder (500 g) of *Cordyceps cicadae* was extracted under reflux with distilled water (2 L × 3) at 100 °C for 2 h. The resulting supernatant were pulled together and concentrated to a specific volume under reduced pressure using a rotary evaporator. The concentrated supernatant was precipitated by adding 4 vol. of cold 50% ethanol (v/v) overnight at 4 °C. The precipitate obtained was centrifuged, washed twice with 95% ethanol and dissolved in distilled water. The solution was lyophilized to obtain the crude polysaccharide. The crude polysaccharide was deproteinized using Sevag method and decolorized using macro-porous resin D101 (Li et al., 2015). The sample was loaded on a DEAE-cellulose column and successively eluted with distilled water, and a gradient of 0.1–0.2 M NaCl at 1.0 mL/min. Each fraction was monitored and assayed for its carbohydrate content using the phenol-sulfuric acid method. The eluates obtained from distilled water and 0.1 M NaCl was subjected to further individual purification using Sephadex-G100 to obtain two polysaccharide's fraction (CPA-1 and CPB-2) were collected, dialyzed and lyophilized.

2.4. Analysis of monosaccharide composition

The monosaccharide composition of the purified CPA-1 and CPB-2 was performed by gas chromatography (GC). The polysaccharides (10 mg each) were hydrolyzed in a sealed glass tube with 2 M trifluoroacetic acid at 100 °C for 4 h. After removing the excess acid, the hydrolysate was acetylated with 5 mg of hydroxyl-amine hydrochloride in 1 mL of pyridine for 1 h at 90 °C. After cooling, acetic anhydride (1 mL) was added and further incubated for 1 h at 90 °C. The derivative was then converted into their corresponding aldononitrile acetate derivatives and analyzed with GC. Composition identification was done by comparison with reference standard (rhamnose, arabinose, xylose, mannose, glucose and galactose).

2.5. FT-IR spectral analysis

CPA-1 and CPB-2 (5.0 mg/each) was ground with KBr and pressed into a pellet. The FT-IR spectra were analyzed on a Perkin-Elmer spectrometer from 4000 to 400 cm-1.

2.6. Cell culture and treatment

PC12 cells were routinely maintained in DMEM containing 10% (v/v) FBS, 100 U/ml penicillin and 100 μg/ml streptomycin at 37 °C in a humidified atmosphere of 95% air and 5% CO_2. The culture medium was changed every other day.

PC12 cells were pretreated with different concentrations of CPA-1 and CPB-2 (25, 50, 100 and 200 μg/ml) for 24 h, and then incubated with 5 mM glutamate for an additional 24 h. The control group was administered with the same amount of DMEM. CPA-1 and CPB-2 were dissolved in DMSO with the final concentration of DMSO less than 0.1% (v/v).

2.7. Determination of cell viability

Cell viability was performed using MTT colorimetric assay. PC12 cells (1.0×10^4 cells/well) were seeded in a 96-well plate. After cell treatment, the medium was removed, and the cells were incubated with 20 μL of 5 mg/ml MTT solution for 4 h at 37 °C. The medium was carefully removed, and the dark-blue formazan was dissolved with 150 μL of DMSO. The absorbance was determined at 490 nm with a microplate reader. Cell viability was expressed as a percentage of control cells.

2.8. LDH activity determination

LDH leakage into the culture medium indicates cell injury. The LDH activity was measured according to the manufacturer's instructions in the assay kit. After the treatment, the media was collected and the supernatant was used in the assay of extracellular LDH activity. The absorbance was measured at 450 nm using a microplate reader. Data represents the percentage of LDH released relative to control cells.