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The core structure of a *Dendrobium huoshanense* polysaccharide required for the inhibition of human lens epithelial cell apoptosis

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ABSTRACT

The aim of this work was to investigate the core structure of a *Dendrobium huoshanense* polysaccharide DHPD1 required for the inhibition of lens epithelial cell apoptosis. In order to obtain the fragments containing the core domain, pectinase was employed to hydrolyze DHPD1. After 24 h reaction, it is interesting that the hydrolyzation seemed to be stopped, leading to a final enzymatic fragment DHPD1-24 with molecular weight about 1552 Da. Compared to DHPD1, although the bioactivity is decreased, DHPD1-24 remained the ability to inhibit the H_2O_2-induced apoptosis of human lens epithelial (HLE) cells via suppressing the MAPK signaling pathways. These results suggested that DHPD1-24 might be the core domain required for DHPD1 to inhibit HLE cell apoptosis. Methylation analysis showed DHPD1-24 was composed of (1 → 5)-linked-Ara*f*, (1 → 3,6)-linked-Man*p*, 1-linked-Glc*p*, (1 → 4)-linked-Glc*p*, (1 → 6)-linked-Glc*p*, (1 → 4,6)-linked-Glc*p*, (1 → 6)-linked-Gal*p* and 1-linked-Xyl*p* in a molar ratio of 1.06:1.53:2.11:2.04:0.93:0.91:0.36:1.01. Moreover, the primary structural features of DHPD1-24 were characterized by NMR spectrum.

1. Introduction

Cataract, a leading cause of blindness worldwide, is characterized as the opacification of eye lens (Vinson, 2006). The World Health Organization survey showed that more than 17 million are blind due to cataract and about 28,000 new cases around the world are reported each day (Vinson, 2006). The elderly population is more susceptible to suffer from cataract, accounting for 25% of people over 65 years and 50% over 80 years. This group is expected to increase four-fold worldwide (Vinson, 2006). Therefore, cataract is a major health disease and a serious social problem, which has brought a heavy economic burden to our society. Currently, surgery is a major method to treat cataract, but accompanied surgical complications can result in visually disabling cataract (Luo, Deng, & Zha, 2008). Recently, there is considerable interest in identifying preventive factors that may delay the onset or progression of lens opacities, and traditional Chinese medicines have received great attention for this purpose (Rahman, 2003).

Dendrobium huoshanense C.Z. Tang et. S.J. Cheng, a famous traditional Chinese medicine, has been used as a therapeutic agent to protect eyesight (Luo et al., 2008). In recent decades, *D. huoshanense* is also reported to be a tonic for promoting the production of body fluid and improving the quality of life (Fang et al., 2015). Our recent findings showed that polysaccharides from *D. huoshanense* (DHP) exhibited good ability to prevent the onset and development of cataract via inhibiting the oxidation pathway *in vivo*, suggesting DHP is the active compounds required for *D. huoshanense* to protect eyesight (Luo et al., 2008). The polysaccharides extracted from cortex of *Paeonia suffruticosa* were also found to have protective effects on diabetic cataract in rats (Zhao, Shen, Ma, Li, & Shi, 2007). However, there is still no report on the structure and mechanisms of polysaccharides with anti-cataract. It is well accepted that there is a specific oligosaccharide fragment (core structure) required for polysaccharide to recognize the target cells and thus exhibit biolog-

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ical activity (Muralikrishna & Rao, 2007; Schepetkin & Quinn, 2006; Zhang, Cui, Cheung, & Wang, 2007). Therefore, to find the specific oligosaccharide fragment is beneficial for unraveling the structural basis of polysaccharides required for biological activity.

To achieve these purposes, pectinase was employed to prepare the oligosaccharide fragment of *D. huoshanense* polysaccharide in the present study. Growing evidences indicated that the occurrence of cataract is highly associated with the apoptosis of lens epithelial cells, attributing to the special structure of this organ (Miyazawa, Takamura, Aoki, Akagi, & Singh, 2003; Charakidas et al., 2005). Therefore, using the apoptosis model of human lens epithelial (HLE) cells induced by H_2O_2, the active specific oligosaccharide fragment was determined, and the structural features and possible mechanism of this fragment were further demonstrated.

2. Materials and methods

2.1. Materials and reagents

D. huoshanense was collected from Huoshan county, Anhui province of China. Protocorm-like bodies (PLBs) of *D. huoshanense* were induced and propagated on Murashige and Skoog medium according to the reference (Qian et al., 2014). Pectinase (EC 3.2.1.15) were purchased from Yuanju Bioscience Technology Co., Ltd. (Shanghai, China). DEAE-cellulose, Sephadex G-25 and Bovine serum albumin (BSA) were obtained from Sigma Chemical Co. (MO, USA). The dimethylsulfoxide (DMSO) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma Chemical Co. (MO, USA). ELISA kits of IL-1β and TGF-β2 were obtained from R&D Co., Ltd. (Nanjing, China). All the primary antibodies were purchased from the Cell Signaling Technology Inc. (MA, USA). The secondary antibody was supplied by Wuhan Boster Co. (Wuhan, China). Dextrans were purchased from Fluka (Gallen, Switzerland). All other reagents used in this paper were analytical grade.

2.2. Preparation of DHPD1 and its enzymatic fragments

Fresh PLBs (100 g) were homogenized and extracted with 300 mL distilled water in a water bath at 50–60 °C for 2 h. After centrifugation at a speed of 12,000 rpm, the supernatant was collected and concentrated to a certain volume at 60 °C on a rotary evaporator. To obtain crude polysaccharides, the liquid concentrates were mixed with ethanol at a final concentration of 80% (v/v) and precipitated at room temperature for 24 h. Sevag method was employed to remove proteins from the precipitates. The precipitates with free of proteins were further dialyzed and lyophilized, giving the crude polysaccharides of *D. huoshanense* (DHP). The crude DHP (100 mg) were purified on a DEAE-cellulose anion-exchange column using double distilled water as the eluent. The obtained eluent were lyophilized to give the purified polysaccharide (DHPD1).

In order to unravel the active core domain of DHPD1, pectinase was employed to hydrolyze DHPD1. The reaction conditions were fixed at enzyme activity 126 U/mL, pH 4.46 and reaction temperature 48 °C. The samples were taken from the reaction mixture periodically for reducing sugar analysis. Based on the dynamic profiles of degradation, the reaction time was determined to be 1 h, 8 h and 24 h, and therefore giving three different hydrolysates. Each hydrolyzate was further successively purified on a DEAE-cellulose anion-exchange column and a gel permeation chromatographic column of sephadex G-25. The final purified products were named as DHPD1-1, DHPD1-8 and DHPD1-24 for 1 h hydrolysate, 8 h hydrolysate and 24 h hydrolysate of DHPD1, respectively. The reducing sugars were determined according to 3,5-dinitrosalicylic acid method (Potumarthi, Baadhe, Nayak, & Jetty, 2013).

2.3. Homogeneity, molecular weight and monosaccharide composition analysis

The homogeneity and molecular weight of polysaccharides were determined by high performance liquid chromatography (HPLC, Agilent Technologies) according to the reference (Xie et al., 2016). The HPLC was equipped with a TSK G4000PWXL column (7.8 mm × 300 mm) and a TSK G5000PWXL column (7.8 mm × 300 mm), which were connected in series. A refractive index detector was employed to recognize polysaccharide. The column temperature was set at 35 °C and a 20 μL aliquot of sample was injected for each run. Double distilled water was used as the eluent at a flow rate of 0.5 mL/min. The columns were calibrated with Dextrans series (31418, 31420, 31422, 31424 and 31425). According to our previous reports (Zha et al., 2012), monosaccharide composition in different polysaccharides was analyzed by gas chromatography (GC).

2.4. Glycosidic bond analysis

The methylation method was performed to analyze the types of glycosidic bond in polysaccharides. According to our previous reports (Qian et al., 2014), after the polysaccharides (20 mg) were fully methylated, the resulting products were hydrolyzed in 2 mL TFA (2 M) at 120 °C, giving methylated monosaccharides. The methylated monosaccharides were further converted into aditol acetates and analyzed by GC–MS (Qian et al., 2014).

2.5. NMR spectroscopy

One hundred milligrams of each polysaccharide were deuterium exchanged several times by lyophilizing from D_2O and then examined as solutions in 99.99% D_2O containing a trace of acetone as internal standard. NMR spectra (^1H NMR, ^{13}C NMR, HSQC and HMBC) were recorded at 27 °C on a Bruker Avance AV400 spectrometer. Data processing was performed using standard Bruker XWIN-NMR software.

2.6. Cell line and cell culture

The HLE cells were purchased from Shanghai Fusheng Industrial Co. Ltd. (Shanghai, China) and cultured in DMEM medium supplemented with 10% fetal bovine serum, 100 U/mL penicillin and 100 μg/mL streptomycin at 37 °C in a humidified incubator with 95% atmosphere and 5% CO_2.

2.7. Analysis of cell viability

The effects of polysaccharides on HLE cell viability were evaluated by MTT assay. The HLE cells were briefly seeded into a 96-well culture plate at the density of 5×10^4 cells/mL and cultured at 37 °C in a humidified incubator with 95% atmosphere and 5% CO_2. After 4 h of incubation, the cells were treated with DHPD1 at a final concentration of 10, 50, 100, 200, 400, 800 and 1600 μg/mL for another 20 h. At the end of incubation, the culture medium was taken out and 5 mg/mL of MTT reagents were added to each well followed by incubation for 4 h at 37 °C. At last, 100 μL of DMSO were added to each well, and the absorbance was recorded at 570 nm on a Bio-Rad model 680 Microplate Reader (PA, USA).

2.8. Effects of polysaccharides on HLE cell viability in the presence of H_2O_2

The HLE cells were seeded into a 96-well plate at the density of 5×10^4 cells/mL and cultured at 37 °C in a humidified incubator with 95% atmosphere and 5% CO_2. After incubation for 4 h, 5 μL