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Antioxidant activity and characterization of antioxidant polysaccharides from pine needle (*Cedrus deodara*)

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ABSTRACT

A novel antioxidant polysaccharide (APC) was isolated and characterized from pine needles of *Cedrus deodara* with the evaluation of its *in vitro* antioxidant activity. According to gel filtration chromatography, high performance size exclusion chromatography, gas chromatography–mass spectrometry, partial acid hydrolysis, periodic acid oxidation, Smith degradation and methylation analysis, APC was observed to be an acidic heteropolysaccharide (composed of glucose, arabinose, mannose and xylose in a molar ratio of 45.84:1:2.35:1.73) with the molecular weight of 1.53×10^4 Da, and the backbone was mainly composed by glucose, mannose and xylose in the form of $(1 \rightarrow 4)$ linked. Meanwhile, APC exhibited the remarkable antioxidant activity to scavenge free radicals and inhibit the oxidative injury of DNA and cells. The present results suggested that APC could be a potential antioxidant agent for preparing functional foods and nutraceuticals applied in food and pharmaceutical industries.

1. Introduction

Reactive oxygen species (ROS) generated in aerobic organisms during respiration process, such as hydroxyl radical ($OH^{\bullet}$), superoxide anion ($O_2^{\bullet -}$) and hydrogen peroxide (H_2O_2), may be necessary for normal function of cells at physiological levels (Page, Moser, Chen, & Dutton, 1999). But, the excessive ROS are harmful to cells, due to their strong attacking capacity to lipids, proteins and deoxyribonucleic acid (DNA), which can cause various cellular damages and diseases, such as oxidative damage of DNA, cardiovascular disease and neurological disease (Martínez-Cayuela, 1995; Fraser, 2011; Gil del valle, 2011). In the last decade, huge interests had received the great attention and had been studied extensively to identify free radical scavengers or antioxidants, which could reduce risks of oxidative damage and several cardiovascular diseases. Although the synthetic antioxidants, such as propyl gallate (PG) and butylated hydroxytoluene (BHT), could inhibit the oxidative damage effectively, their potential toxic in human body was being considered and worried (Carocho, & Ferreira, 2013). Thus, many efforts had been made to find the novel and safe antioxidants from natural source (Ng, Liu, & Wang, 2000; Podsędek, 2007; Karre, Lopez, & Getty, 2013).

Polysaccharides, one essential biomacromoleules of life activities, are usually composed of various monosaccharides linked with different glucosidic bonds, which widely exist in plants, animals and microorganism. Being associated with proteins and polynucleotides, polysaccharides participate in cell adhesion, signal recognition, molecular recognition and cell–cell communication, and also play important roles in the immunization, reproduction and blood systems (Dwek, 1996; Gilbert, Knox, & Boraston, 2013). Recently, many bioactive polysaccharides obtained from different natural source were observed to exhibit various biological activities, such as immunopotentiation, reducing the blood glucose and blood triglycerides, antitumor, anticoagulant, antiirradiation and antioxidant activities, which have attracted much attention in the field of biochemistry and pharmacology (Jin, Zhao, Huang, Xu, & Shang, 2012; Cao, 2013). Furthermore, a lot of studies had been made to elucidate the relationship between structures and biological effects of polysaccharides, and the accumulated evidence demonstrated that structure of polysaccharides had the significant influence to their biological activities (Yang & Zhang, 2009; Sun, 2011).

Cedrus deodara, commonly named Himalayan cedar, belongs to *Pinaceae* and widely grows in Asia, such as China, India, Japan and Korea. With the significant nutritional and pharmaceutical effects, pine needles of *C. deodar* have been widely used in food industry to

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produce beverage, and in herbal medicine to treat tic, fever, cough, bronchitis and tuberculosis (Fang, Quan, & Cai, 2010; Chaudhary, Ahmad, & Mazumder, 2011; Bai, Shi, Liu, & Li, 2012). Recently, it has been reported that water-extract of *C. deodara* pine needles exhibited the remarkable antibrowning, antioxidant and antibacterial activities, which was due to its high content of phenolic compounds (Zeng et al., 2011; Zeng, He, Sun, Zhong, & Gao, 2012). Furthermore, its essential oil was observed to possess the excellent antioxidant and antimicrobial capacity (Zeng, Zhang, Gao, Jia, & He, 2012). Therefore, pine needles of *C. deodar* might have the potential value to be utilized as a novel nutraceuticals to protect human health.

To the best of our knowledge, the investigation about the characterization and corresponding antioxidant activity of polysaccharides from *C. deodara* pine needles is rather limited. Therefore, efforts have been paid to characterize a novel antioxidant polysaccharide from *C. deodara* pine needles, and its antioxidant activities were also determined detailedly in present study. Furthermore, we attempted to observe its protective effects on the oxidative injury of DNA and cells induced by hydrogen peroxide.

2. Materials and methods

2.1. Materials and reagents

Pine needles of *C. deodara* were collected from Chengdu of China in July, 2012. The plant was initially identified by the morphological features, and morphological data were kept in the Department of Biology, Sichuan University. A voucher specimen was dried and preserved at the Department of Food Engineering, Sichuan University. *C. deodara* pine needles were washed and dried at 45 °C for 12 h, and then were crushed into powders (about 60 granularities) with a mixer (JYL-350, Jiuyang Co., Ltd., China), and finally stored under vacuum.

2,2′-Azinobis-3-ethylbenzthiazoline-6-sulphonate (ABTS), pyridine, inositol, acetic anhydride, 1,1-diphenyl-2-picrylhydrazyl (DPPH), butyl hydroxy anisd (BHA), nitro blue tetrazolium (NBT), dihydronicotineamidadenine dinucleotide (NADH), phenazine methosulfate (PMS), ethylene diamine tetraacetic acid (EDTA), trifluoroacetic acid (TFA) and thiobarbituric acid (TBA) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Plasmid pBR322 DNA was purchased from Takara Bio-medicals (Tokyo, Japan). A Chinese hamster lung fibroblast cell line (V79 cells) was purchased from Chengdu Biological Institute (Chengdu, China). The solvents for high performance size exclusion chromatography (HPSEC) and gas chromatography–mass spectrometry (GC–MS) were of chromatographic purity. All other reagents were of analytical grade. The water used in all test was treated in a Milli-Q water purification system (Millipore, Bedford, MA, USA).

2.2. Extraction of crude polysaccharides

The pine needle powders (2000 g) were added to 5000 mL of distilled water with continuously stirring at 85 °C for 45 min. Then, the mixture was filtered and the extraction procedure repeated twice. Thereafter, all supernatants were condensed using a rotary evaporator (R-II, Kejinyu, Co. Ltd., Zhengzhou, China) at 45 °C under vacuum. Bing treated with Sevage reagent and dialyzed (MWCO 5000, Sigma–Aldrich, USA), crude polysaccharides were precipitated with 5-fold volumes of absolute alcohol, and then were prepared by centrifugation and freeze-drying. The yield of crude polysaccharides was 3.58% (3.58 g crude polysaccharides/100 g dried pine needle powders).

2.3. Purification and characterization of antioxidant polysaccharides

2.3.1. Isolation and purification

Crude polysaccharides (10 g) were separated on the DEAE-cellulose 52 column (3 cm × 50 cm), Sephadex G-50 gel filtration column (3 cm × 75 cm) and Toyopearl HW-65F column (3 cm × 70 cm), and eluted using a linear gradient of 0–1 M NaCl solution at a flow rate of 0.2 mL/min to arrive at homogenous preparation. The polysaccharides obtained at this stage were designated as "APC". The total saccharide content and quantification of uronic acid of APC were determined according to the phenol-sulfuric acid assay (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956) and the vitriol-carbazole method (Yang et al., 2008), respectively.

2.3.2. Determination of homogeneity, molecular weight and monosaccharide composition

According to the analytical methods in our previous study (Zeng, Zhang, Gao, Jia, & Chen, 2012), the homogeneity and molecular weight of APC were determined by high performance size exclusion chromatography (HPSEC) using an Agilent 1100 HPLC system (Agilent Technologies, Ltd., CA, USA) and a size exclusion chromatography (SEC) column (TSK gel Super SW 2000, 4.6 mm × 300 mm i.d. with a particle size of 4 μm, Tosoh, Tokyo, Japan), and the monosaccharide composition of APC was analyzed by gas chromatography–mass spectrometry (GC–MS).

2.3.3. Partial acid hydrolysis, periodic acid oxidation, Smith degradation and methylation analysis

The structure properties of APC, such as the link model and sequence of monosaccharide, was characterized by using the analytical tests of partial acid hydrolysis, periodic acid oxidation, Smith degradation and methylation analysis according to the methods in our previous study (Zeng, Zhang, Gao, Jia, et al., 2012).

2.4. Evaluation of the free radical scavenging activity

The free radical (ABTS, DPPH, superoxide and hydroxyl radicals) scavenging activity of APC was evaluated according to the detailed methods in our previous study (Zeng, Zhang, Gao, Jia, et al., 2012). APC was dissolved in distilled water at different concentrations (0.25–4 mg/mL) for the measurements.

2.5. Protection on the oxidative damage of DNA induced by hydrogen peroxide

The protective capacity of APC on the oxidative damage of DNA induced by hydrogen peroxide was determined as the procedure described by Yeung et al. (2002) with some modifications. Briefly, 3 μL of phosphate buffer (50 mM, pH 7.4) containing 0.5 μg of pBR322 DNA, 3 μL of EDTA–$FeSO_4$ solution (2 mM) and 2 μL of APC solution at various concentrations (20–320 μg/mL) were mixed. Then, 4 μL of 30% H_2O_2 were added and the mixture was incubated in water bath at 37 °C for 1 h, which was conducted in an Eppendorf tube. When the reaction was completed, the mixture was subjected to 1% agarose gel electrophoresis. DNA bands (supercoiled, linear, and open circular) were stained with ethidium bromide and quantified by scanning the intensity of bands with quantity one program (version 4.2.3, BioRad Co.). Protective effect on DNA was based on the increase or loss percentage of supercoiled monomer, comparing with control. To avoid the effects of photoexcitation of samples, all assays were done in the dark.