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Structure characterization and biological activities of a pectic polysaccharide from cupule of *Castanea henryi*

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ABSTRACT

A pectic polysaccharide (CHIP3) was fractionated from the natural cupule of *Castanea henryi*. It contained mannose (10.70%), rhamnose (8.70%), galacturonic acid (38.21%), galactose (13.75%) and arabinose (28.63%) with a molecular weight of 2.44×10^4 g/mol by multi-laser light scattering. The structure was elucidated by using FT-IR spectroscopy, methylation analysis and NMR analysis. Results showed that the backbone of CHIP3 consisted of 1, 4-α-linked D-Gal*p*A residues containing the non-methyl-esterified carboxyl groups, interspersed with a few 1,2-α-L-Rha*p* units. Its side chains were attached by two branches to O-4 of Rha*p* with 1,4-β-linked D-*Galp* units and 1,5-α-L-linked Ara*f* units bearing 3,5-substituted α-L-linked Ara*f* residues as branching points. AFM data revealed it existed as a flexible chain in 0.1 M $NaNO_3$ aqueous solution. Furthermore, CHIP3 was demonstrated to have notable antioxidant activity of FRAP, $ABTS^{\bullet+}$ radical scavenging and reducing power. Cytotoxicity assay showed it displayed inhibitory activity against HepG2 cells with IC_{50} values of 242.6 μg/mL.

1. Introduction

Plant acidic polysaccharides, particularly pectic polysaccharides, are widely distributed in the roots, stems, leaves, fruits etc. of primary cell walls of all land plants [1]. According to the features of their structure, there are four types of pectic polysaccharides: homogalacturonan (HG), rhamnogalacturonan I (RG-I), rhamnogalacturonan II (RG-II), and xylogalacturonan (XGA) [2]. High-molecular pectic polysaccharides have excellent gelling and emulsifying properties, and have been used as thickeners, gelling agents, emulsifiers and stabilizers in the food industry [3,4]. Low-molecular or modified pectic polysaccharides exhibit good biological activities, due to low molecular weight, low degree of esterification, good solubility, easily absorbed into the intestine [5–8]. Although only apple pomace and citrus peel are the most common commercial sources of pectin [9], other novel sources, such as amaranth, alfalfa, potato, *Panax ginseng*, etc. [5,10–13], have also attracted researcher's attention. Pectic polysaccharides are excellent sources for widely applying in pharmaceutical, cosmetics, and food industries, and study on those with immunomodulatory or antitumor activity from plant resources has become a hot research topic.

Castanea henryi, belongs to Fagaceae Castanea, is a traditional dried fruit with considerable economic value. In addition to being a popular green food, it has been used as a traditional Chinese medicine for a long time [14]. Therefore, there are abundant resources of *Castanea henryi* in China, whose deep processing industry is extremely well-developed, but their cupule with burs of 1–2 cm long are usually being discarded upon harvesting, resulting in a waste of resources. As far as we know, previous studies have mainly focused on the part of fruits [14,15], little information on its cupule. In this regard, the elucidation of structure and biological activities of the components from the cupule of *C. henryi* is necessary to better utilize these tons of wastes.

In this study, a pectic polysaccharide from the cupule of *C. henryi* was extracted by hot-water and subsequently isolated and purified by column chromatography. The structural and molecular morphological features of the purified fraction were then characterized by HPLC, FT-IR, methylation, GC–MS, 1D and 2D NMR spectroscopy, size exclusion chromatography combined with a multi-angle laser light scattering (SEC-MALLS) and atomic force microscopy (AFM)

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analyses. Further, its antioxidant and antitumor activities were evaluated *in vitro* as well.

2. Materials and methods

2.1. Materials and chemicals

The *Castanea henryi* cupule was obtained from qingyuan county, Zhejiang Province, China. DEAE-52 cellulose, Sephacryl S-200 was purchased from the GE Healthcare Ltd. (Chalfront St. Guiles, UK). Papain, standards of monosaccharides, PMP (1-phenyl-3-methyl-5-pyrazolone), NaN_3, BSA, Ascorbic acid (Vc), were purchased from the Sinopharm Chemical Reagent Co. (Beijing, China). TPTZ [1,3,5-tri(2-pyridyl)-2,4,6-triazine], 2,2′-azino-bis-(3-ethylbenzothiazoline-6–sulfonic acid) diammonium salt (ABTS), 2,2′-azobis (2-methylpropionamidine)-dihydrochloride (AAPH), fluorescein sodium salt, 6-hydroxy-2,5,7,8 -tetramethylchroman-2-carboxylic acid (Trolox) were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). All other reagents used were of analytical grade.

2.2. Extraction, fractionation and purification of pectic polysaccharide

The dried powder of *Castanea henryi*'s cupule (through a 200-mesh screen) was defatted by ethanol (95%, v/v) extraction at 90 °C for 3 h, and then extracted by hot distilled water (90 °C, three times) for 3 h with the ratio of 1:20 (m/v). After filtration and concentration, the resulting aqueous solution was precipitated using 4-fold volumes of ethanol (95%, v/v) for a day at 4 °C. The precipitates were collected by centrifugation at 8000 rpm for 10 min, and then re-dissolved in distilled water, freeze-dried to yield crude polysaccharides, named as CHIP. In order to remove protein within CHIP, it was subsequently dissolved in distilled water (2%, m/v), treated with Papain (2%, m/v), 60 °C, pH 6.0, 4 h and fully oscillated with one-third volume of Sevag reagent (chloroform:butanol = 4:1, v/v). Then prepared polysaccharide was obtained by dialysis and lyophilization.

CHIP was dissolved in distilled water (10 mg/mL), filtered through 0.45 μm membrane and then loaded to diethylaminoethyl (DEAE)-52 column (2.0 cm × 35 cm). It was eluted with distilled water and a stepwise elution of 0.1 M, 0.3 M, 0.5 M, 0.7 M and 1.5 M NaCl at a flow rate of 1.0 mL/min. Eluate was collected automatically with 6 mL for each tube and monitored by the phenol-sulphuric acid method at 490 nm [16]. The major polysaccharide fraction was collected, dialyzed, lyophilized and further purified by Sephacryl S200 column (1.5 cm × 60 cm, GE Healthcare), which was eluted by 0.05 M PBS (pH 7.4) contained 0.15 M NaCl at a rate of 0.5 mL/min, and with collection of 3 mL for each tube. Each eluting peak was collected, dialyzed thoroughly and lyophilized to yield a pure polysaccharide, named as CHIP3.

2.3. Monosaccharide composition

3 mg of CHIP3 was dissolved in 1 mL of 2 mol/L TFA in a sealed tube, and hydrolyzed for 6 h kept at 121 °C. After being completely hydrolyzed, the excess TFA was dried under nitrogen at 70 °C by adding 200 μL methanol for three times. The hydrolysate was dissolved in 1 mL of distilled water for further derivatization. Monosaccharide compositions of CHIP3 were prepared by pre-column derivatization with PMP and identified by RP-HPLC according to the method of Zhang et al. [17] with a proper modification. Briefly, 100 μL of standard monosaccharides (2 mM) or the hydrolysates of CHIP3 were mixed with 100 μL of 0.6 M aqueous NaOH and 100 μL of 0.5 M PMP in methanol solution. Subsequently, the mixture was bathed in water at 70 °C for 30 min, and neutralized with 100 μL of 0.3 M HCl after cooling to room temperature. The resulting solution was added to 1 mL, extracted with the same volume of chloroform to remove PMP, and repeated twice. Finally, the aqueous layer was filtered through 0.45 μm filter membrane prior to HPLC analysis.

Analysis of the PMP-labeled monosaccharides was performed on a Waters 2695 HPLC system (Waters, US) equipped with a PDA 2996 detector (Waters, US). The analytical column used was a Zorbax Eclipse XDB-C18 column (4.6 mm × 250 mm, 5 μm, Agilent, USA). Elution was carried out at a flow rate of 1.0 mL/ min at 25 °C, which contained 15% 0.05 M sodium phosphate (KH_2PO_4-NaOH, pH 6.8) buffer and 40% acetonitrile, applying a linear gradient as follow: 0 → 10 min → 30 min → 35 min → 40 min, corresponding to buffer B 0 → 15% → 25% → 25% → 0. The wavelength of UV detection used was 250 nm. The injection volume was 10 μL.

2.4. Study of infrared (FT-IR) spectroscopy

The major structural information of the purified CHIP3 was performed using a Fourier-transform infrared spectrophotometer (Nexus IS10 FTIR, Thermo Nicolet, USA). The CHIP3 sample (2 mg) was pressed into KBr pellet at sample: KBr ratio 1:20. The Fourier transform-infrared spectra were recorded in the range of 4000–400 cm^{-1} and processed by Omnic software [18].

2.5. Methylation analysis

CHIP3 was first reacted with $NaBD_4$ to reduce the uronic acid residues following the procedure described eariler [19]. Then methylation of carboxyl-reduced CHIP3 was conducted as described by Ciucanu and Kerek [20] with some proper modification. In brief, 2 mg of CHIP3 was prepared in 1 mL anhyd DMSO (dimethylsulfoxide) and sonicated for a while under nitrogen. Afterwards the derivatization was triggered by adding 1 mL of cold CH_3I dropwise in 0.6 mL NaOH-DMSO solution under nitrogen until it was fully cooled. The resulting solution was kept to react for 30 min in the ultrasonic bath. Complete methylation was confirmed by the disappearance of the O—H band (3200–3700 cm^{-1}) in the IR spectrum. Then the permethylated sample was treated with 3 mL of 90% aqueous formic acid for 3 h at 100 °C for depolymerization in a sealed tube. After removing the residues of formic acid, the depolymerized fragments were hydrolyzed with 2 M trifluoroacetic acid (4 mL) for 6 h at 100 °C in another sealed tube. Finally, the methylated alditol acetates were analyzed by gas chromatography-mass spectrometer (GC–MS) after fully reduction with $NaBH_4$ and acetylation with 1-methyl imidazole and acetic anhydride. GC–MS was carried out on Agilent 7890A/5975C instrument with a TG-5MS column (30 m × 0.25 mm × 0.5 mm) (Thermo Co., Austin, TX, USA) equipped with flame-ionization detector. The temperature was increased from 120 to 220 °C at a rate of 5 °C/min and then maintained at 220 °C for 15 min. The injector and the detector temperatures were both set at 250 °C.

2.6. Nuclear magnetic resonance (NMR) spectroscopy

40 mg of CHIP3 was dried for 48 h in a vacuum dryer with P_2O_5 and then dissolved in 0.5 mL D_2O and lyophilized three times for replacing exchangeable protons. 1D/2D ^1H and ^{13}C NMR spectra of polysaccharide solution were recorded on a Bruker AVANCE 600 MHz spectrometer (Bruker Group, Fäl-landen, Switzerland) at 333.15 K with the operating frequency of 600 MHz, which were assigned by ^1H NMR, ^{13}C NMR, ^1H-^1H COSY, TOCSY, HSQC and HMBC. Chemical shifts were referenced tointernal DSS (^1H and ^{13}C at 0.00 ppm). Data was obtained and analyzed using standard Bruker NMR software.