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Antioxidant and immunoregulatory activity of alkali-extractable polysaccharides from North American ginseng

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ABSTRACT

The alkali-extractable polysaccharide (AEP) was isolated from the root of North American ginseng. Two fractions, AEP-1 and AEP-2, were further purified by gel filtration column chromatography. Gas chromatography analysis identified that AEP-1 was composed of Glc, Gal and GalA. And AEP-2 mainly contained Ara, Man, Gal, Glc and GalA. Antioxidant assays indicated that AEP and AEP-2 exhibited significant antioxidant activities in a dose-dependent manner. AEP-2 also exhibited macrophage-activating activity by increasing NO, TNF-α and IL-6 production. The results suggest that AEP-2 could be used as potential antioxidants and immunomodulators.

1. Introduction

North American ginseng (*Panax quinquefolium* L.), a plant native to North America, has been recognized as a valuable tonic similar to Asian ginseng (*Panax ginsen* C.A. Meyer) [1]. North American ginseng is reported to have many activities, such as anticarcinogenic, antiaging, immunostimulatory and antioxidant effects [2]. Various phytochemicals were found to be responsible for the health benefits of North American ginseng. Ginsenosides and polysaccharides in North American ginseng were recognized as the two major active components [3]. Many studies had proved that polysaccharides in North American ginseng showed strong immunoregulatory effect [4–7]. However, most of the reported polysaccharides were prepared from water extraction of North American ginseng. And, after water extraction, the residue was usually abandoned. Actually, there are still some components in the residue which are of great use, such as alkali-extractable polysaccharides. As far as we know, there was no report about the extraction and purification of alkali-extractable polysaccharides from North American ginseng, as well as the antioxidant and immune activity determination.

In this work, we isolated alkali-extractable polysaccharides from North American ginseng, identified their chemical characteristics, and determined the antioxidant and immunomodulatory effects.

2. Materials and methods

2.1. Materials and regents

Four-year-old roots of North American ginseng were obtained from Fusong County (Jilin Province, China). DEAE Sepharose Fast Flow and Sephacryl S-300 High Resolution were obtained from GE Healthcare Bio-Sciences Co. (Piscataway, NJ, USA). Monosaccharide standards including galacturonic acid (GalA), glucose acid (GlcA), glucose (Glc), rhamnose (Rha), arabinose (Ara), galactose (Gal), xylose (Xyl) and mannose (Man) were purchased from Sigma–Aldrich (Shanghai, China). DMEM, RPMI 1640 medium, lipopolysaccharide (LPS), Griess reagent, sodium fluorescein (3′,6′-dihydroxyspiro[isobenzofuran-1[^{3}H],9′[^{9}H]-xanthen]-3-one, FL), 2,2′-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) radicals, 2,2′-azobis (2-amidinopropane) dihydrochloride (AAPH), and 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) were also purchased from Sigma–Aldrich. Fetal bovine serum (FBS) was obtained from GE Healthcare Bio-Sciences (Piscataway, NJ, USA). Raw murine macrophages (RAW 264.7) were purchased from National Platform of Experimental Cell Resources for Sci-Tech (Beijing, China). All other chemicals and solvents used were of analytical grade unless otherwise specified.

2.2. Extraction, separation, and purification of polysaccharide

The dried roots of North American ginseng were ground into powder. The powder passing through an 80 mesh sieve was

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extracted as follows. Step 1, removal of ethanol-soluble substances: The powder was extracted twice with 15 times of 70% EtOH at 50 °C for 5 h to get the residue-1. Step 2, removal of water-soluble polysaccharide: the dried residue-1 was extracted twice with 14 times of distilled water at 100 °C for 4 h to get the residue-2; Step 3, extraction of alkali-soluble polysaccharide: residue-2 was extracted twice with 10 times of 0.3 M NaOH solution which contained 0.3% (w/w) $NaBH_4$ at room temperature for 4 h. The supernatant was collected and adjusted to neutrality with hydrochloric acid (1 M), and then dialyzed, concentrated, and deproteinated by Sevag method [8]. The supernatant containing alkali-extractable polysaccharide (AEP) was precipitated with ethanol (1:4 v/v) at 4 °C for 24 h and then lyophilized to get AEP.

AEP was purified on an ÄKTA explore 100 purification system. AEP was dissolved in distilled water, centrifuged (15,000 rpm, 20 min), and then the supernatant was loaded on a DEAE Sepharose Fast Flow column (2.6 cm × 100 cm) equilibrated with ultrapure water. The column was first eluted with distilled water, then with a linear gradient from 0 to 2.0 M NaCl at a flow rate of 4 mL/min. Different fractions (8 mL/tube) were collected using an automatic fraction collector, then dialyzed and lyophilized. The fractions were purified further on a Sephacryl S-300 High Resolution column (1.6 cm × 100 cm) eluted with 0.15 M NaCl at a flow rate of 0.5 mL/min to yield two main final fractions, named AEP-1 and AEP-2, respectively. The fractions obtained were combined according to the total carbohydrate content quantified by the phenol-sulfuric acid method [9].

2.3. Analysis of monosaccharide composition

AEP-1 and AEP-2 were hydrolyzed by trifluoroacetic acid (2 M) at 120 °C for 4 h, respectively [10]. Derivatization of the released monosaccharides was carried out by the trimethylsilylation reagent according to the method of Guentas et al. [11]. The trimethylsilylated derivatives was further analyzed by gas chromatography (GC) on an Agilent 6890 instrument (Agilent Technologies, USA) equipped with HP-5 MS capillary column (0.25 mm × 30 m × 0.25 μm) and determined by a flame ionization detector (FID). The following program was adopted for gas chromatography analysis: injection temperature: 250 °C; detector temperature: 260 °C; column temperature programmed from 100 to 150 °C at 5 °C/min, holding for 5 min at 150 °C, then increasing to 240 °C at 5 °C/min and finally holding for 3 min at 240 °C. Nitrogen was used as the carrier gas and maintained at 40.0 mL/min. The speed of air and hydrogen gas was 400 and 40 mL/min, respectively. The injection was in spiltless mode.

2.4. Infrared spectrum analysis

Fourier transform infrared (FT-IR) spectra were obtained by using a PerkinElmer FT-IR spectrometer (PerkinElmer Spectrum 400 FT-IR, Massachusetts, USA) at the range of 4000–500 cm^{-1}.

2.5. Assays for antioxidant activity

2.5.1. Trolox equivalent antioxidant capacity assay

The method used was as described by Re et al. (1999), based on the capacity of a sample to inhibit the ABTS radical compared with a reference antioxidant standard (Trolox) [12]. Briefly, ABTS radical solution was produced by reacting 7 mM of ABTS aqueous solution with 2.45 mM potassium persulphate, and the mixture was kept in the dark at room temperature for 16 h. Then ABTS radical solution was diluted with PBS (pH 7.0) to an absorbance of 0.70 (±0.02) at 734 nm. Each sample (0.2 mL) with various concentrations (0.0–5.0 mg/mL) were added into 2 mL of ABTS radical solution and mixed vigorously. The mixture solution was incubated for 6 min at room temperature. The absorbance was measured at 734 nm. The antioxidant activity of crude polysaccharide extracts and polysaccharide fractions was measured by Trolox equivalent antioxidant capacity (TEAC) analysis as described by Fukumoto and Mazza (2000) [13].

2.5.2. Oxygen radical absorbance capacity assay

The oxygen radical absorbance capacity (ORAC) assay was conducted according to the method of Ou et al. with some modifications [14]. The ORAC assay was carried out on a FLUOstar OPTIMA plate reader (BMG LABTECH GmbH, Offenburg, Germany). The ORAC assay employs the area under the curve of the magnitude and time to the oxidation of fluorescein due to $ROO^{\bullet}$ radicals generated by the addition of AAPH in the presence of added antioxidants [15]. FL and AAPH were prepared with 75 mM phosphate buffer at pH 7.4. Samples (25 μL) at different concentrations (0–5 mg/mL) were mixed with 100 μL of 7.98×10^{-4} mM FL and incubated at 37 °C for 10 min than added 75 μL of 173 mM AAPH. Fluorescence was collected with 485 nm excitation and 515 nm emission, taking measurements from each sample at 2 min intervals for 2 h. The inhibition capacity was expressed as Trolox equivalents (μmol Trolox/g), and is quantified by integration of the area under the curve (AUC). All determinations were performed in triplicate.

2.6. Assay for immunomodulatory activity

2.6.1. Nitric oxide (NO) production

Nitric oxide (NO) production was assayed by measuring the nitrite concentration in the supernatant of cultured macrophages using the Griess reaction [16]. Briefly, cells were incubated in medium alone (control group) or medium containing various concentrations of polysaccharides fractions (1, 10, 50, 100, 150 μg/mL) or lipopolysaccharide (LPS, 1 μg/mL) as a positive control. Cells were incubated at 37 °C in 5% CO_2 for 24 h, and then the supernatants (50 μL) were pipetted from the medium and mixed with an equal volume of Griess reagent. After incubation for 15 min at room temperature, the absorbance was measured at 540 nm in an ELISA reader (Rayto RT-6000, Shenzhen, China). The concentration of nitrite was calculated with reference to a standard curve obtained with $NaNO_2$ (0–100 μM).

2.6.2. Measurement of cytokine production

RAW264.7 cells were cultured at a density of 2×10^5 cells/well for 24 h with polysaccharide fractions (10, 50, 150 μg/mL) or LPS (1 μg/mL), the control group was treated with medium alone. The supernatants were collected for the detection of tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6) production using commercial ELISA kit (BD Biosciences Pharmingen, San Diego, USA) according to the instructions of kits. The absorbance was measured at 450 nm and 570 nm in an ELISA reader. Cytokine quantities in the samples were calculated from standard curves of recombinant cytokines using a regression linear method.

3. Results and discussion

3.1. Extraction and purification of alkali-extractable polysaccharide

AEP was isolated from the root of North American ginseng and the yield was about 2.85%. The purification was performed on a DEAE Sepharose Fast Flow column to obtain water-eluted and salt-eluted fractions, accounting for 64.2% and 25.1% of AEP by weight, respectively (Fig. 1a). And then based on molecular weight difference, the two fractions were further purified using a column of Sephacryl S-300. Each fraction yielded only one peak (Fig. 1b and c),