International Journal of Biological Macromolecules 56 (2013) 1–5

Contents lists available at SciVerse ScienceDirect

International Journal of Biological Macromolecules

journal homepage: www.elsevier.com/locate/ijbiomac

Antioxidant activity of polysaccharide fractions extracted from *Athyrium multidentatum* (Doll.) Ching

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ARTICLE INFO

Article history:
Received 18 December 2012
Received in revised form 18 January 2013
Accepted 19 January 2013
Available online 25 January 2013

Keywords:
Athyrium multidentatum (Doll.) Ching
Antioxidant activity
Polysaccharide
Radical scavenging activity
Chelating effect

ABSTRACT

Crude polysaccharides were extracted from *Athyrium multidentatum* (Doll.) Ching (AMC) rhizome and fractionated by DEAE-Cellulose 52 ion-exchange column chromatography. Two polysaccharide fractions (F1 and F2) were obtained and had their antioxidant activities investigated employing various established in vitro systems. All fractions possessed considerable antioxidant activity. Chemical analysis suggested that F1 and F2 were neutral heteropolysaccharide in which glucose was the major component. Available data suggested that the molecular weight and sulfate content played very important roles on antioxidant activity. Our results indicated that the polysaccharides may contribute to the medicinal functions of AMC.

1. Introduction

The concept of reactive oxygen species (ROS) has gained significant recognition over the past several years by various studies in laboratories worldwide. ROS are chemically reactive molecules containing oxygen, including superoxide anion radical ($^{\bullet}O_2^-$), hydroxyl radical ($^{\bullet}OH$), hydrogen peroxide (H_2O_2), singlet oxygen ($^1O_2$), and nitric oxide ($NO^{\bullet}$). ROS form as a natural by-product of the normal metabolism of oxygen and play important roles in cell signaling and homeostasis [1]. However, excessive ROS can induce generation of oxidative stress and lead to cell damage [2]. Oxidative stress is considered to be a primary factor in diseases such as diabetes, cancer, rheumatoid arthritis, and atherosclerosis as well as in degenerative processes of aging [3,4]. Antioxidants can reduce the oxidative stress by breaking the oxidative chain reaction, and protect the body from oxidative damage. However, some commonly used synthetic antioxidants, such as BHA and BHT, are restricted by legislative rules because they are suspected to have some toxic effects and as possible carcinogens [5]. In recent few decades, considerable interest has arisen in finding non-toxic antioxidants from natural sources [6].

Athyrium multidentatum (Doll.) Ching (AMC), a species of fern in the Athyriaceae family, distributes widely in the Northeast and Shandong of China. AMC is a popular potherb of Changbai Mountain area and contains plenty of nutrients, such as protein, carbohydrates, fats and vitamins, which benefit health in many ways. Furthermore, it has been used as a drug in traditional Chinese medicine for tranquilization, lowering blood pressure and diuresis, etc. Our preliminary study has shown that polysaccharides extracted from AMC is active as an antioxidant, being strong in scavenging superoxide radical and reducing power [7]. There are many reports in the literature on the antioxidant capacities of polysaccharides. However, not all carbohydrates act as antioxidant. Antioxidant activities of polysaccharides depend on sugar unit, glycosidic bonds in the main chain, the types and polymerization degree of the branch, flexibility and configuration of the chains [8]. Nevertheless, no detailed studies have been carried out on the correlation between chemical characteristics and antioxidant properties of AMC polysaccharides.

In this study, two polysaccharide fractions were prepared and their in vitro antioxidant activities were evaluated using two different ROS scavenging assays containing superoxide/hydroxyl radical scavenging activity, and chelating ability. The purpose of the present study was to investigate the antioxidant activity and characterize the relationship between chemical property and antioxidant activity.

Abbreviations: AMC, *Athyrium multidentatum* (Doll.) Ching; ROS, reactive oxygen species; BHA, butylated hydroxyanisol; BHT, butylated hydroxytoluene; CPA, crude polysaccharides of AMC; H_2O_2, hydrogen peroxide; TCA, trichloroacetic acid; Vc, vitamin C; Gal, galactose; Man, mannose; Glc, glucose; Ara, arabinose; Rha, rhamnose; PMP, 1-phenyl-3-methyl-5-pyrazolone; EC_{50}, efficient concentration defined as the concentration inhibiting 50% radical generation or scavenging 50% radical generated.

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http://dx.doi.org/10.1016/j.ijbiomac.2013.01.023

2. Materials and methods

2.1. Chemicals

DEAE-Cellulose 52 was purchased from Whatman International Ltd. Pyrogallol, trichloroacetic acid (TCA), hydrogen peroxide (H_2O_2), ferrozine, and vitamin C (Vc) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Other reagents were of analytical grade and purchased from China National Pharmaceutical Group Co. All chemicals and reagents, unless otherwise specified, were not purified, dried or pretreated. *A. multidentatum* (Doll.) Ching rhizome, cultured in Changbai Mountain area, Jilin, China, was harvested in September 2010. The fresh rhizome was washed thoroughly, air-dried and kept in plastic bags at room temperature for use.

2.2. Preparation of crude polysaccharides

A total of 600 g of AMC rhizome was crushed and refluxed in 4800 mL water for 3 h. The hot solution was separated from AMC residues by successive filtration through gauze and siliceous earth. The solution was concentrated to about 350 mL under reduced pressure, and then added 1100 mL of anhydrous ethanol. The resultant precipitate was filtrated, dried, and dissolved in distilled water (5%, mass fraction). Insoluble substances were separated and discarded by centrifugation. Free proteins were removed from crude polysaccharides using Sevag method [9]. Crude polysaccharides of AMC (CPA) were obtained and kept in vacuum desiccator for use (mean yield 2.9%).

2.3. Further fractionation

Further fractionation was performed using anion-exchange column chromatography. Crude polysaccharides (3.0 g) were dissolved in distilled water and applied to a DEAE-Cellulose 52 column (4.0 cm × 50 cm) pretreated with HCl and NaOH. Fractions were prepared in stepwise elution with increased concentration of NaCl (0.5, 1.0, 1.5, and 2.0 M) solution at a flow rate of 50 mL/h. The elution was detected by phenol–sulfuric acid method [10]. Each fraction was dialyzed against tap water for 48 h and against distilled water for 24 h using 3500 Da M_w cutoff dialysis membranes, and finally lyophilized in a freeze dryer (BeiJing Bilon Lab. Equipment Co., Ltd., China).

2.4. Chemical analysis

Total sugar content of each fraction was determined according to the method of Du et al. [11], using glucose as standard. Sulfate content was analyzed by barium chloride–gelatin method [12]. Molecular weights were determined by high performance size exclusion chromatography. A gel-filtration chromatographic column of TSK G3000SW (Bio-Rad, Richmond, CA, USA) was used and maintained at 35 °C. Samples were filtered through 0.45 μm filter membrane before analysis. The injection volume was 20 μL and the mobile phase was 2.84% (w/v) Na_2SO_4 buffer with flow rate of 0.5 mL/min. The detection was carried out at 35 °C with a refractive index detector. Preliminary calibration of the column was conducted using dextrans of different molecular weights. Multichrom with GPC software (Thermo Lab-Systems, Shrewsbury, MA, USA) was used to acquire and analyze the molecular weight data.

2.5. Determination of monosaccharide component

Monosaccharide compositions were quantified by HPLC method as described by Yang et al. [13]. In brief, polysaccharide samples were hydrolyzed in a sealed glass tube with 2 M trifluoroacetic acid at 110 °C for 6 h. The excess acid was completely removed at 70 °C by a steady stream of nitrogen. The hydrolyzed products were derivatized in distilled water containing 0.3 mol/L NaOH and 0.5 mol/L 1-phenyl-3-methyl-5-pyrazolone (PMP) at 70 °C for 1 h, and then neutralized with 0.3 mol/L HCl. Finally, the solution was extracted with $CHCl_3$ three times. PMP derivatives were filtered through 0.45 μm filter membrane and analyzed by HPLC using a XDB-C18 column (4.6 mm × 150 mm, 5 μm, Agilent). The injection volume was 10 μL. The chromatographer was monitored by UV absorbance at 245 nm and run isothermally at 30 °C. The phosphate buffer/acetonitrile (83:17, v/v) was used as mobile phase with flow rate of 1.0 mL/min. As references, the following standard monosaccharides were derived and analyzed accordingly: L-rhamnose, L-fucose, L-arabinose, D-xylose, D-mannose, D-galactose, D-glucose, D-glucuronic acid, D-galacturonic acid, D-glucosamine, and D-galactosamine.

2.6. Determination of antioxidant activity

2.6.1. Superoxide radical scavenging assay

Measurement of superoxide radical scavenging activity was based on a modified method of Zhang et al. [14]. Superoxide radicals were generated in the pyrogallol autoxidation system containing 4.0 mL Tris–HCl buffer (50 mM, pH 8.2), 0.5 mL pyrogallol (3.5 mM), and varying concentrations of samples (4.0–20.0 μg/mL). The reaction mixture was incubated at room temperature for 6 min. The reaction was terminated by 0.5 mL HCl (8.0 M). Absorbance was measured at 320 nm against the blank. In the control, sample was substituted with Tris–HCl buffer. Measurements were performed at least in triplicate. Decreased absorbance of the reaction mixture indicated increased superoxide anion scavenging activity. The scavenging capacities of samples were compared with Vc. The capability of scavenging to superoxide radical was calculated using the following equation:

$$\text{Scavenging effect}\ (\%) = \left(1 - \frac{A_{\text{sample}\,320}}{A_{\text{control}\,320}}\right) \times 100$$

2.6.2. Hydroxyl radical scavenging assay

Hydroxyl radical scavenging ability of sample was assessed by a modified method of Cao et al. [15]. The reaction mixture, containing H_2O_2 (1.0 mM) and $FeSO_4$ (2.0 mM), was incubated with salicylate (6.0 mM) at 37 °C for 15 min. Varying concentrations of samples (10.0–50.0 μg/mL) were added and incubated at 37 °C for another 15 min. Hydroxyl radical was detected by monitoring absorbance at 510 nm. In the control, sample was substituted with distilled water. The hydroxyl radical inhibition percentage was calculated according to the given formula:

$$\text{Scavenging effect}\ (\%) = \left(1 - \frac{A_{\text{sample}\,510}}{A_{\text{control}\,510}}\right) \times 100$$

2.6.3. Metal chelating assay

The ferrous ion-chelating ability of sample was investigated with slightly modified method described by Zhang et al. [14]. Samples in different concentrations (5.26–26.31 μg/mL) were mixed with 1.0 mL $FeCl_2$ (2.0 mM) and 0.2 mL ferrozine (5.0 mM), shook well, stayed still for 10 min at room temperature, and then the absorbance of the mixture was determined at 562 nm. In the control, sample was substituted with distilled water. The chelating capacity of sample was compared with EDTA. The ferrous ion-chelating activity was given by the following equation:

$$\text{Chelating ability}\ (\%) = \left(1 - \frac{A_{\text{sample}\,562}}{A_{\text{control}\,562}}\right) \times 100$$