Carbohydrate Polymers 187 (2018) 19–25

Contents lists available at ScienceDirect

Carbohydrate Polymers

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

The isolation, structural characterization and anti-osteosarcoma activity of a water soluble polysaccharide from *Agrimonia pilosa*

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ARTICLE INFO

Keywords:
Agrimonia pilosa
Polysaccahride
Osteosarcoma
Apoptosis
Mitochondria

ABSTRACT

A homogenous polysaccharide (APP), with a molecular weight of 120 kDa, was isolated from the dried aerial parts of *Agrimonia pilosa*. Gas chromatography (GC) and GC–MS analysis revealed that APP has a backbone of 1,3-linked Glcp and 1,3, 6-linked Glc*p*, and branched with 1-linked Glc*p* terminal along the main chain in a relative ratio of 2:1:1. We investigated the response of human osteosarcoma U-2 OS cells to APP treatment. MTT result showed that APP significantly inhibited cell viability in a concentration dependent manner via induction of apoptotic death in U-2 OS cells, as determined by annexin V/propidium iodide (PI) staining. Western blot analysis also indicated that APP CRA increased in Bax/Bcl-2 ratios by up-regulating Bax expression and triggered the release of cytochrome c from mitochondria into the cytoplasm. Moreover, APP supplement induced the activation of caspase-3, and −9, but not caspase-8 in U-2 OS cells. Likewise, APP administration significantly suppressed tumor growth in BALB/C nude mice bearing U-2 OS xenograft tumors. All these results indicate that APP-induced apoptosis is associated with the activation of a caspase-3-mediated mitochondrial pathway.

1. Introduction

Osteosarcoma is the most common primary malignant bone tumour and occurs predominantly in childhood and young adults (Jemal et al., 2008). The overall 5-years survival rate of osteosarcoma patients was 10% before the 1970s when treatment was mainly limb amputation (Sun et al., 2011). For limb salvage, the principles of treatment of osteosarcoma have undergone dramatic changes in the past 20 years (Dass, Ek, Contreras, & Choong, 2006; Longhi, Errani, De Paolis, Mercuri, & Bacci, 2006). Until recently, the rate dramatically increased to approximately 65%, since the introduction of multi-agent chemotherapy followed advanced surgery (Ferrari et al., 2005). Hence, with the advent of chemotherapy, the prognosis of osteosarcoma patients and long-term survival significantly improved. However, most chemotherapeutics carry the risk of severe toxicity and/or lack of efficacy. Adriamycin (ADM) has been used most widely as anti-osteosarcoma drug, but its value is limited by toxicity, which arouses a great concern (Vermorken, 2003). As a consequence, various new chemotherapeutics that are affordable and have minimal toxicity are presently being evaluated in clinical trials around the world.

Recently, the use of natural products from medicinal plants in modern medicine has been regarded as one of the most important aspects for cancer control (Jo et al., 2004). For this reason, it is of importance to explore novel antitumor agents present in medicinal plants that can inhibit the progression of cancer. Among the naturally occurring substances, polysaccharides are a class of biological macromolecules with tremendous structural diversity that have proven to be useful candidates in the search for effective, non-toxic substances with therapeutic effects and thus their pharmacological properties have attracted substantial attention in medicine industry (c Ooi & Liu, 2000; Sinha & Kumria, 2001). Natural polysaccharides have been reported to have many pharmacological activities including antitumor, immunomodulatory, antioxidant, antimicrobial, antiulcer, and so on (Sun et al., 2008). Among them, antitumor effects are of high priority, and numerous antitumor polysaccharides have been found to be non-toxic to normal cells and can induce apoptosis of various cancer cells (Chen et al., 2013; Li et al., 2014; C. Li et al., 2015; Wang et al., 2013). *Agrimonia pilosa* LEDEB. (Rosaceae) is a perennial herb, which is distributed primarily in temperate regions of East Asia (Kim et al., 2016). The aerial parts of this plant have been used as a traditional medicine for treatment of urinary, respiratory, digestive system, and bleeding diseases in the Chinese Pharmacopoeia (Wang, Liu, Wei, Yan, & Jin, 2012; Xu, Qi, Wang, & Chen, 2005). It possess diverse interesting biological activities including anti-oxidant (Zhu et al., 2009), anti-inflammatory, anti-allergic activity (Kim et al., 2012), acetylcholinesterase inhibition (Jung & Park, 2007), anti-nociception

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https://doi.org/10.1016/j.carbpol.2018.01.047
Received 12 December 2017; Received in revised form 12 January 2018; Accepted 16 January 2018

activity (Park et al., 2012) and anti-tumor activity (Li, Li, Tip, & Zhang, 2015; Miyamoto, Kishi, & Koshiura, 1987). Previous investigations of the chemical constituents of *A. pilosa* have shown the presence of various possible bioactive compounds, such as flavonoids, polyphenol, lactone, organic acid, triterpenoide and acid polysaccharide (Liu, Hou, Chen, Liang, & Sun, 2016; Zhang, Dai, & Zheng, 2008). However, to our knowledge, no report exists on the antitumor activity of netural water soluble polysaccharide from *A. pilosa* on human osteosarcoma cell line. Considering the popularity of natural products from the Chinese herbal medicine commonly used as adjunctive agent in clinical for cancer therapy, we aim to evaluate the effectiveness of a polysaccharide from *A. pilosa* on cell growth and apoptosis in human osteosarcoma U-2 OS and normal human osteoblast NHOst cell line, and the observed antitumor activities were also verified in a xenografted mouse model in vivo.

2. Materials and methods

2.1. Materials and chemicals

A. pilosa was purchased from the local Drug store in Wuhan city of China. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), Dimethyl sulfoxide (DMSO), glucuronic acid, glucose and trifluro acetic acid (TFA) was purchased from Sigma–Aldrich (St. Louis, MO, USA). T-series dextran of consisting of T-2000 (MW: 2,000,000), T-500 (MW: 473,000), T-70 (MW: 102 67,200), T-40 (MW: 43,000), T-10 (MW: 10,000) was from Beijing Baier Di Biotechnology Co., Ltd. (Beijing, China). DEAE cellulose and Sepharose CL-6B were purchased from Pharmacia Co. (Sweden). Antibodies against Bax, Bcl-2, and cytochrome c were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The caspases activity assay kits were from kaiji Bio-Tek Corporation (Nanjing, China). Caspase-3 inhibitor (Z-DEVE-FMK), caspase-8 inhibitor (Z-IETD-FMK), and caspase-9 inhibitor (Z-LEHD-FMK) were from R&D systems (Minneapolis, MN, USA) and were dissolved in DMSO and diluted in cell culture medium before use. Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), penicillin, streptomycin, L-glutamine and gentamicin were purchased from Invitrogen (Carlsbad, CA). Annexin V-fluorescein isothiocyanate (FITC)/propidiumiodide (PI) kit were obtained from Pharmingen-Becton Dickinson (San Diego, CA, USA). All other chemicals (Na_2SO_4 $NaBH_4$, acetic anhydride, pyridine) were obtained from commercial sources and were of analytical grade.

2.2. Extraction, isolation and purification of polysaccharide from A. pilosa

The dried aerial parts of *A. pilosa* (290 g) was cut into pieces, and extracted with 10.0 l of 95% ethanol for 3 times and 3 h for each time at 100 °C to eliminate lipids and some colored materials. The pre-treated *A. pilosa* was then successively boiled with 15 l of distilled water under reflux for 2 h each time and 3 times. The suspension (35.6 l) collected by centrifugation (8000 × *g* for 30 min) was concentrated in a rotary evaporator under reduced pressure of 40 psi at 50 °C, and the free protein (1.5 g) in this concentrate was removed by Sevag agent (isoamyl alcohol and chloroform in 1:4 ratio) (Staub, 1965), followed by exhaustive dialysis against tap water and distilled water each for 48 h. After centrifugation (1700 × *g* for10 min) at 20 °C, the supernatant (12.4 l, 34.53% of original supernatant volume) was concentrated 8-fold (1.55 l) and a 5-fold volume (7.75 l) of 95% ethanol was added to the aqueous extracts to precipitate the crude polysaccharides at 4 °C overnight, giving 13.2 g of crude polysaccharide (CAPP, 4.55% of raw material).

Each 80 mg crude CAPP was dissolved in 1.5 ml distilled water and filtered through a membrane (0.45 μm, Nucleopore), and then the solution was fractionated on a DEAE-cellulose column (Cl^- form, 2.5 cm × 50 cm) and eluted successively with distilled water and stepwise gradient of NaCl aqueous solutions (0.2, 0.6, and 1.0 M) at a flow rate of 4.0 ml/min. Fractions of 8 ml were collected and monitored by phenol–sulfuric acid method. After dialysis and lyophilization, the fraction eluted with distilled water was loaded onto a Sepharose CL-6B column (100 cm × 2 cm) and eluted with 0.15 M NaCl at a flow rate of 1.0 ml/min to yield one major fraction. This fraction was pooled, concentrated, desalted and lyophilized to obtain a purified polysaccharide named APP.

2.3. Physicochemical characteristics, molecular weight (Mw) and monosaccharide composition of the polysaccharide

Total sugar content was determined by phenol-sulfuric acid method, with glucose as the standard (DuBois, Gilles, Hamilton, Rebers, & Smith, 1956). The protein contents were quantified according to the Bradford's method (Bradford, 1976). Total uronic acid contents were assayed using the carbazole-sulfuric acid method with glucuronic acid as the standard (Filisetti-Cozzi & Carpita, 1991).

The homogeneity and average Mw of the isolated polysaccharide APP were estimated on Waters high performance liquid chromatography (HPLC) system with TSK-G3000 PWXL column (7.8 mm ID × 30.0 cm), a Waters 2410 differential refractive index detector (RID-10A) and an on-line degasser. The sample was applied and eluted with 0.1 M Na_2SO_4 solution in PBS buffer (0.01 M, pH 6.8) at a flow rate of 0.6 ml/min. Commercially available Dextran T-series standards were used as standard molecular markers to plot the calibration curves. The retention time of APP was substituted into the equation of the calibration curve to calculate the Mw.

The monosaccharide composition of APP was determined using gas chromatography (GC) analysis. Briefly, APP (10 mg) was hydrolyzed with 2 M TFA at 100 °C for 6 h to hydrolyze and release the component monosaccharides. The acetylated samples conventionally derived from the monosaccharides were analyzed by GC on an Agilent 7890N GC instrument (Santa Clara, CA, USA) equipped with flame ionization detector (FID) and a HP-5 fused silica capillary column (0.25 mm i.d. × 30 m × 0.25 μm) (Jones & Albersheim, 1972; Oades, 1967).

2.4. Methylation analysis

2 mg of sample was completely methylated twice according to the method of Anumula and Taylor (1992). Complete methylation was monitored by the disappearance of the OH stretching band ranging from 3200 to 3700 cm^{-1} in the IR spectrum. Afterwards, the methylated polysaccharides were hydrolyzed with 2 mol/L TFA (4 ml) at 120 °C for 2 h, followed by reduction with $NaBH_4$ for 24 h and acetylation with acetic anhydride–pyridine (1:1) at 100 °C for 2 h. The resulting partially methylated alditol acetates were separated by the chloroform–water system and finally identified by GC–MS to determine the glycosyl linkage.

2.5. Cell cultures

Human osteosarcoma cell line U-2 OS and normal human osteoblast cell line NHOst were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). All cells were cultured in DMEM supplemented with 10% FBS, 100 U/mL penicillin G, and 100 μg/mL streptomycin, 1 mM L-glutamine and 100 μg/mL gentamicin at 37 °C under a humidified atmosphere containing 5% CO_2 at 37 °C.

2.6. Cell viability

Cell viability was determined using a MTT assay, which was based on the conversion of MTT to MTT-formazan by mitochondrial enzymes (Carmichael, DeGraff, Gazdar, Minna, & Mitchell, 1987). Briefly, U-2 OS cells or NHOst were seeded into 96-well culture plates at a density of 3×10^3 cells per well in 200 μl of medium in triplicate and incubated for 24 h. After overnight incubation, APP ranging from 25–100 μg/mL